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THE SEROLOGICAL IDENTIFICATION OF *CL. TETANI*.

J. D. MACLENNAN.

From the Department of Bacteriology, University of Aberdeen, and the Department of Pathology, British Postgraduate Medical School.

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IT has been stated in a recent survey (Gay, 1935) that for the accurate identification of *Cl. tetani* at least two of three criteria should be satisfied. The organism should conform morphologically and culturally with known strains of *Cl. tetani*, it should produce typical tetanic spasms in animals, and it should agglutinate to approximately full titre with a serum prepared against one of the serological types of *Cl. tetani*.

Although in practice such a system of identification may be useful, it must be evident that it is far from being completely adequate. Aberrant morphological and cultural variants have been described in recent years by Heller (1922), Condrea (1928), Manzini (1931) and others; non-toxigenic strains are now known to be comparatively common (Fildes, 1927, 1929); and the fact that nine serological types of *Cl. tetani* have so far been identified probably indicates that there are many more awaiting recognition.

With the exception of Type VI, which is non-motile, the separation of *Cl. tetani* into serological types depends entirely on the flagellar antigens. It seemed possible, therefore, that a solution to the problem might lie in the heat-stable components of the species. In the present paper are recorded the results of various investigations into the O antigens of *Cl. tetani*; at the same time an attempt has been made to confirm the findings of others with regard to the subdivision of *Cl. tetani* into nine serological types on the basis of the flagellar antigens. Also, an account is given of an organism which on serological grounds should be classified as *Cl. tetani*, but which does not fall into any of the recognized serological types—apparently being a new type. While this work was in progress Gunnison (1937) published the results of an extensive study of the heat-stable antigens of *Cl. tetani*. Briefly, she found

that there was an O antigen common to all nine types ; in addition to this, toxigenic strains of Type II, IV, V and IX possessed a further heat-stable component.

For the present investigation 23 strains of *Cl. tetani* were employed—three of each of Types I–VII, and one each of the rare Types VIII and IX. All strains were toxigenic.

MATERIAL AND METHODS.

The bacterial suspension used for the production of H sera in rabbits was prepared in the following manner : The type-strain of *Cl. tetani* was cultured anaërobically in heart broth for from 24 to 48 hours ; the bacterial cells were then spun down, washed, resuspended in saline to an opacity corresponding to 2000×10^6 *Bact. coli* per c.c., and heated to 60° C. for 1 hour to destroy all traces of toxin. It was generally advisable to repeat this heating 24 hours later, as further toxin frequently appears in the suspension. Fildes (1925) has already noted the occurrence of this phenomenon. As a rule 3 intravenous injections of 0·5 c.c. of this vaccine at 3-day intervals were sufficient to produce high titre antisera at the end of a fortnight.

For the preparation of O antisera, a similar suspension of cells was heated in the steamer at 100° C. for 1 hour, and then readjusted to the same opacity. Half a c.c. of the resulting suspension, injected intravenously on alternate days for 6 to 7 injections, always gave satisfactory antisera.

For the absorption of antisera, 0·5 to 1·0 c.c. of the packed cells was added to 5 c.c. of a 1/5 dilution of the antiserum and incubated at 37° C. overnight. The bacteria were then removed by centrifugalization. It was occasionally found necessary to perform these absorptions with two successive doses of cells.

The agglutination tests were carried out with the bacterial suspension diluted to an opacity of 500×10^6 *Bact. coli* per c.c. The antisera were pipetted out in 0·3 c.c. amounts in serial dilutions from 1/20 to 1/2560. An equal amount of the bacterial suspension was added, giving final dilutions of the serum from 1/40 to 1/5120. The reaction was read after 4 hours at 56° C., and again after standing overnight at room temperature.

RESULTS.

Flagellar Antigens.

The findings with regard to the H agglutinins may be dismissed briefly, since in all essential respects they confirm those of Tulloch, Bauer, Fildes, Coleman and others. The reaction is of a peculiarly massive and flocculent character, usually appearing within a few minutes of setting up the test. The antisera tend to be quite specific, with little overlapping between heterologous types. There is some slight cross-agglutination to a titre of 1/160 or less between Types I and VII, V and IX, and notably IV and V. This has already been recognized, and can readily be circumvented by means of adequate absorption. The Type VI strains (all of which are non-motile) show a very fine agglutination. This does not appear until 3 hours, and is not at all marked

until 5 or 6 hours. Furthermore, the Type VI strains agglutinated to a variable extent with any of the other 8 type-sera. As this cross-agglutination was also of a fine and granular nature it was thought probable that it was due to residual somatic agglutinins. Accordingly specimen antisera of each type were absorbed with successive doses of cells of all the other types ; such sera contained no O agglutinins and were extremely type-specific ; they would not agglutinate the Type VI strains even in a titre of 1/20. The unabsorbed H antisera were then tested against boiled O suspensions of all nine types. They agglutinated such suspensions in a manner and to a titre exactly comparable to those obtained with the Type VI strains. It is evident, therefore, that Type VI, so-called, is not a true serological type, but rather a dumping-ground for all non-motile variants.

Somatic Antigens.

In contrast to the flagellar, the somatic agglutination, as in the case of other organisms, is very slow in appearing (2 to 3 hours at the earliest), and is of a particularly fine and granular character. The actual degree of this granularity is not constant, however, and seems to vary with the strain employed in the preparation of the suspension and with the amount of heating to which it has been subjected.

An O antiserum prepared against any type of *Cl. tetani* would agglutinate suspensions prepared from any of the nine types to high titre ; indeed, sometimes the titre was higher with heterologous than with the homologous types. Unheated suspensions of *Cl. tetani* either would not agglutinate with the O antisera or would agglutinate only to a very low titre. In any case the reactions with such suspensions were always irregular and unsatisfactory. The three Type VI strains, however, gave consistently good results even if unheated, though usually to a slightly lower titre than the heated antigens. It is to be emphasized that for accurate work the O suspensions should be absolutely fresh, as they tend to deteriorate very rapidly, and after a month are more or less inagglutinable. There is no tendency to auto-agglutination such as Condrea (1928) has described as occurring with his heated suspensions.

Absorption experiments.

By absorption experiments the findings of Gunnison are confirmed. There is an O antigen common to all nine types, and in addition Types II, IV, V and IX possess a further O antigen common to themselves. It seemed possible that these results might indicate quantitative rather than qualitative differences between the O antigens of the various types ; but repeated absorption of the antisera of Types II, IV, V and IX with heated suspensions of Types I, III, VI, VII and VIII invariably failed to remove all agglutinins from the absorbed antisera. It seems extremely probable, therefore, that another O antigen is present in certain types of *Cl. tetani*, quite distinct from the common somatic antigen. I did not possess non-toxigenic strains of Types II, IV, V and IX, and cannot confirm the statement of Gunnison that such variants do not possess the additional component.

Atypical Tetanus Bacillus H 9.

While the above investigations were still in progress a considerable number of anaërobes with terminal spores were received from Dr. Shewan of the Marine Biological Research Station at Torry. These, together with many more anaërobic bacilli isolated by him from the intestines of various North Sea fish, are the subject of a recent communication (Shewan, 1938). All the strains received were identified more or less readily with the exception of one organism —H 9. Morphologically this was indistinguishable from a typical tetanus bacillus. It was a slender Gram-positive rod, soon becoming Gram-negative, motile by means of numerous peritrichous flagella, and forming, after 48 hours, a large round terminal spore. Culturally and biologically it differed from *Cl. tetani* in only one respect. This was in the character of its growth on agar and blood-agar. Even without careful drying of the plates, H 9 exhibited no tendency to spread in a film ; instead, within 48 hours there appeared small, coarsely-fluffy colonies with a ground-glass centre. These colonies were altogether much coarser than those of any of the strains of *Cl. tetani* that I have succeeded in growing discretely. The organism failed to climb up an agar or blood-agar slope in the manner described by Fildes, and utilized by him in the isolation of *Cl. tetani*. It should be noted, however, that this property is certainly not found in all strains of *Cl. tetani*, not even in strains which are perfectly typical in all other respects.

The organism was quite non-pathogenic for laboratory animals, even in large doses.

Antigens of H 9.

H and O antisera against H 9 were prepared in the usual manner in rabbits, and the organism and representatives of all serological types of *Cl. tetani* tested out against each other, with the results shown in Tables I, II and III.

TABLE I.—*The Flagellar Agglutinin Reactions of H 9.*

Antigen tested. H suspension.	Titre of H antiserum of Type I.	Titres of H antisera of Types II–IX.	Titre of H antiserum of H 9.
Cl. tetani—Type I	1 : 5120	0	1 : 80
,, ,, II–IX	0	1 : 2560 to 1 : 5120	0
H 9	1 : 40	0	1 : 5120

TABLE II.—*The Somatic Agglutination Reactions of H 9.*

O antisera.	Titre with homologous strain.	Titre with O suspension of H 9.						
		1 : 40.	1 : 80.	1 : 160.	1 : 320.	1 : 640.	1 : 1280.	Control.
Type I	1 : 640	++	++	++	++	+	−	−
,, II	1 : 320	++	++	+−	−	−	−	−
,, III	1 : 1280	++	++	++	++	+	−	−
,, IV	1 : 640	++	++	++	+	−	−	−
,, V	1 : 1280	++	++	++	+	±	−	−
,, VI	1 : 1280	++	++	++	++	++	−	−
,, VII	1 : 320	++	++	++	+	−	−	−
,, VIII	1 : 320	++	++	+−	−	−	−	−
,, IX	1 : 1280	++	++	++	+	+	−	−
H 9	1 : 640	++	++	++	++	+	−	−

TABLE III.—*The Somatic Agglutination Reactions of H 9.*

O antigens of *Cl. tetani.*	Titre of H 9 O antiserum.						
	1 : 40.	1 : 80.	1 : 160.	1 : 320.	1 : 640.	1 : 1280.	Control.
Type I	++	++	++	+	+	−	−
,, II	++	++	++	+−	−	−	−
,, III	++	++	++	++	+	−	−
,, IV	++	++	+	+	±	−	−
,, V	++	++	++	++	+	−	−
,, VI	++	++	++	++	++	−	−
,, VII	++	++	++	+	−	−	−
,, VIII	++	++	++	+−	−	−	−
,, IX	++	++	++	+	+	−	−
H 9	++	++	++	++	+	−	−

Absorption experiments.—Absorption tests with H 9 and with all types of *Cl. tetani* were then carried out. Absorption of H 9 O antiserum with O antigens of any type of *Cl. tetani* removed from the serum all agglutinins both for H 9 and for *Cl. tetani*. The O antisera of the nine types of *Cl. tetani* were then absorbed with heated suspensions of H 9 with the results shown in Table IV.

TABLE IV.—*Absorption of O Antisera with H 9.*

Antigens tested. O suspension.	Titres of O antisera of Types I, III, VI, VII and VIII.		Titres of O antisera of Types II, IV, V and IX.	
	Unabsorbed.	Absorbed with H 9.	Unabsorbed.	Absorbed with H 9.
Cl. tetani, Type I	1 : 640	0	1 : 640	0
,, ,, II	1 : 320	0	1 : 320	1 : 320
,, ,, III	1 : 1280	0	1 : 1280	0
,, ,, IV	1 : 640	0	1 : 640	1 : 320
,, ,, V	1 : 1280	0	1 : 1280	1 : 640
,, ,, VI	1 : 1280	0	1 : 1280	0
,, ,, VII	1 : 320	0	1 : 320	0
,, ,, VIII	1 : 320	0	1 : 320	0
,, ,, IX	1 : 1280	0	1 : 1280	1 : 640
H 9	1 : 640	0	1 : 640	0

DISCUSSION.

From these results it follows that, while the flagellar agglutinogens of H 9 are type-specific, the organism possesses a somatic antigen-complex identical to that found in the majority of toxigenic and all non-toxigenic strains of *Cl. tetani*. It must further be added that, in the course of an inquiry into the antigenic constitution of the anaërobes with terminal spores in general, H and O antisera were prepared against the following organisms: *Cl. putrificum* (3 serological types), *Cl. cochlearium*, *Cl. tetanomorphum* (2 serological types), *Cl. capitovale* (2 serological types), *Cl. paraputrificum*, *Cl. tertium* (3 serological types), *Cl. sphenoides*, *Cl. caloritolerans* and *Cl. putrefaciens*, and that none of these bacilli possessed somatic antigens identical to those found in *Cl. tetani*. It is true that there is some cross-agglutination between *Cl. tetanomorphum* and *Cl. tetani*, but this is to a low titre—1/160 or less—and can always be

removed by suitable absorptions, leaving species-specific antisera. This fact has already been commented on by Gunnison.

In view of these findings it seems justifiable to identify H 9 as a hitherto unrecognized serological type of *Cl. tetani*—Type X.

This method of identifying *Cl. tetani* by means of cross-absorption experiments with suitably absorbed O antisera would seem to have distinct, if limited, possibilities. It is not suggested that it has any universal application—after all, only about a hundred strains of *Cl. tetani* have been examined at all fully from this aspect—yet the technique described should prove of value in a certain number of cases.

The majority of the organisms utilized in the present study were obtained from the collection of Prof. John Cruickshank, of the University of Aberdeen, or were isolated by myself; additional strains have been obtained from Dr. J. M. Shewan, from Dr. D. W. Henderson, and notably from Prof. Ivan C. Hall, to whom my best thanks are due. It is a pleasure to record here my indebtedness to Prof. Cruickshank for much help and advice.

SUMMARY.

1. The H and O agglutinin reactions of *Cl. tetani* have been examined, and the findings of Gunnison (1937) confirmed.

2. The O antigens of *Cl. tetani* are apparently species-specific, and have not been found in any of nine other species of terminally-spored anaërobes.

3. By means of suitable O antisera a new serological type of *Cl. tetani*—Type X—has been identified.

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A VIRUS-INACTIVATING AGENT FROM HUMAN NASAL SECRETION.

F. M. BURNET, DORA LUSH AND A. V. JACKSON.*

From the Walter and Eliza Hall Institute, Melbourne.

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IN this paper experiments are reported dealing with the power of serous nasal secretion from normal human beings to inactivate influenza and certain other viruses. The work originated from certain experiments on the intranasal administration of living egg-adapted virus as a possible means of human immunization. Two individuals with low serum antibody, who had previously been given the " Melbourne " avirulent strain, were sprayed intranasally with egg-adapted " W.S." virus, which is still moderately virulent for ferrets and mice. It was expected that either a clinical attack of influenza or a definite antibody rise would result ; neither in fact occurred. This suggested the possibility that some local protective mechanism was active, and an attempt was made to see whether nasal secretion from these persons possessed virucidal properties. It was soon found that, although their nasal secretion was quite strongly virucidal, so also was that of all the other normal individuals tested.

Although we can find no present indication that this activity of nasal secretion is of any significance in protecting against infection with influenza and other viruses, we felt that the phenomenon justified a fairly extensive investigation.

METHODS.

The technique of obtaining nasal secretion has varied somewhat during the course of the work. The general principle is to absorb the secretion in a roll of gauze or gauze and cotton-wool pushed into each nostril. With many individuals, and particularly in children, it is only necessary to push the plug up high enough to cause moderate discomfort in order to obtain a well-soaked plug in half to one hour. Other persons have been less responsive, and we have used a small amount of 50 per cent. glycerol saline sprayed into each nostril with an atomizer as a mild stimulus to secretion. The plugs are taken out when they become uncomfortably wet and wrung out over an open petri dish by twisting between two artery forceps. As a rule about a cubic centimetre of clear non-viscous fluid is obtained. This is centrifuged and rendered sterile either

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by heating to 56° C. for 30 minutes, or by filtration through a gradocol membrane.

The tests for virus inactivating power are made by mixing equal volumes of nasal exudate and virus suitably diluted in saline containing 10 per cent. of normal horse-serum, and incubating at 36° C. for some hours, usually 5 hours. Control mixtures of dilute virus and saline are also exposed to the same conditions. The mixtures are tested for their virus content on the chorio-allantoic membrane or in the appropriate experimental animal. The reaction of the mixtures used, unless otherwise stated, is approximately pH 8·0.

EXPERIMENTS.

The Action of Nasal Secretion on Influenza Virus Strains.

Filtered nasal exudate inactivates all strains of influenza virus which we have tested. As a first example of the type of result obtained, an experiment with four well-known mouse-passage strains, "Melbourne", W.S.", "Burr" (Smith and Andrewes, 1938), and "Swine 15" was made (Table I). In each case the virus was prepared by grinding one glycerolated lung with 5 c.c. broth and using the spun supernatant fluid. The nasal exudate was a pooled sample from several individuals, and had been filtered through a gradocol membrane. Equal volumes of nasal secretion and virus undiluted or diluted 1/10 were mixed, and after 5 hours at 36° C. each mixture was inoculated intranasally in three young mice. As a control a 1/1000 dilution of each stock virus was left at 36° C. for the same period and also inoculated into mice.

TABLE I.—*Action of Pooled Nasal Secretion on Four Strains of Influenza Virus.*

Strain.	Virus dilution.		Control virus. 1/1000.
	Undiluted.	1/10.	
Melbourne	0, 0, 0	0, 0, 0	4^5, 3, 2
W.S.	2, 1, 1	1, 1, 0	4^5, 4^6, 4^6
Burr	1, 1, 1	0, 0, 0	2, 1, 1
Swine	1, 1, 0	0, 0, 0	3, 3, 3

In this and subsequent similar tables, three mice were inoculated with each mixture and the figures represent the degree of consolidation present in each mouse lung when killed 5 to 7 days after inoculation.

4 = Death with complete consolidation on day shown by the index figure.
3 = More than half the lung consolidated.
2 and 1 = Lesser degrees of consolidation.
0 = No consolidation.

In most experiments the "Melbourne" strain was used, the usual result with undiluted nasal secretion and the standard conditions of contact being to obtain a reduction to about 1/10,000 of the original activity. Individual tests were made on material from 8 adults and 5 children. Only slight differences in activity were found, and for nearly all our work we have used pooled material.

Characteristics of the Virus-inactivating agent (V.I.A.).

The process by which nasal exudate inactivates influenza virus differs essentially from the interaction between virus and antibody. The latter reaction is a rapid one, which, except with very dilute serum, soon reaches an equilibrium level even in the cold, and persists at this level for some time (Burnet, Keogh and Lush, 1937). The action of V.I.A., on the other hand, proceeds much more slowly, and at refrigerator temperature there is little effect in 5 hours. At 36° C. very little inactivation is evident after 30 minutes' contact, and the degree of inactivation increases progressively after 2 and 5 hours' incubation. In Table II the activity of filtered nasal exudate and of serum from the same individual are compared to demonstrate these points.

Table III represents the protocol of an experiment to show (1) the absence of activity of V.I.A. heated to 100° C. and (2) the greatly reduced activity when V.I.A. and virus mixtures are kept at 0° C. instead of at 36° C. The heated material was prepared by immersing a sealed ampoule in boiling water for ten minutes.

It may be noted that there is very little heat-coagulable protein present in the filtered nasal exudate, only a faint opalescence appearing on boiling. Taken with the absence of activity at 0° C., this makes it certain that the effect is not due to traces of serum antibody.

When comparative estimations of the activity of a given preparation are made against mouse-passage and egg-passage influenza virus, there is greater apparent inactivation shown by the mouse tests. As an example, the same filtrate whose activity against " Melbourne " mouse virus is shown in Table I, when tested against "Melbourne" egg virus on the chorio-allantois gave the following figures after the times of contact shown, at 36° C. :

Immediate	90 per cent.
Half-an-hour	12 ,,
2 hours	2·2 ,,
5 hours	0·26 ,,

The figures represent the percentage of foci produced by nasal exudate-virus mixtures in terms of those produced by virus plus saline.

An interesting feature of the results with egg titrations has been the failure of the " percentage law " (Andrewes and Elford, 1933). To take the example just cited, a virus diluted 1/10 with undiluted exudate gave an average of 3·6 foci, indicating a reduction to 0·26 per cent. Undiluted virus, however, with the same exudate, gave semi-confluent membranes, probably carrying about 300 foci, equivalent to 2·2 per cent. of the control. This discrepancy has been fairly regularly encountered, and suggests that V.I.A. is used up in the process of inactivation, perhaps by the soluble virus antigen known to be present in stock egg virus (Hoyle and Fairbrother, 1937). There is no evidence that inactivated virus can be reactivated by dilution in the way that is possible with serum-virus mixtures.

TABLE II.—*Comparison of Filtered Nasal Exudate and Serum from the same Individual against the Influenza Strain "Melbourne" in Mice.*

Agent.	Time of contact at 36° C.	Virus dilutions. Undiluted.	1/10.	1/100.	1/1000.
Filtered nasal exudate	Immediate	..	..	3+, 3, 2	..
,, ,, ,,	1 hour	..	..	3, 2, 1	..
,, ,, ,,	2 hours	3, 3, 2	0, 0, 0	0, 0, 0	..
,, ,, ,,	5 ,,	2, 1, 0	0, 0, 0	..	..
Serum	2 ,,	2, 1, 1	0, 0, 0	..	..
,,	5 ,,	2, 2, 2	0, 0, 0	..	..
Saline	5 ,,	4^4 4^5, 3+	4^5, 3, 3	3+, 3, 3	3, 2, 1

TABLE III.—*Action of V.I.A. on Influenza Virus, "Melbourne" Mouse Strain.*

Inactivating agent.	Time.	Temperature.	Virus dilutions. 1/2.	1/10.	1/100.	1/1000.	Percentage of virus activity remaining.
Filtered V.I.A.	5 hours	36° C.	0. 0, 0, 1 ,1	0, 0, 0, 0, 0, 0	..	..	<0·05
,, ,,	5 ,,	0° C.	..	..	3, 3, 3	2, 2, 2	20
V.I.A. heated 100° C.	5 ,,	36° C.	..	4^5, 4^6, 4^7	3, 3, 2	..	10
Saline	5 ,,	36° C.	..	..	4^6, 4^7, 3	3+, 3, 2	..

Activity of V.I.A. against Other Viruses.

We have also tested by similar methods each of the other viruses which are available in this laboratory for their susceptibility to the agent in nasal exudate. Tests on the egg membrane showed that the viruses of vaccinia, fowl-pox, rabbit myxomatosis, ectromelia of mice, infectious laryngo-tracheitis of fowls and pseudorabies were completely unaffected by the agent, the count of foci obtained falling within a range 50 per cent. $\pm$ of that of control suspensions in saline. Ectromelia and psittacosis viruses were tested by intraperitoneal injection of mixtures into mice. The end-point of infection and the survival times were not significantly different from those obtained with virus-saline mixtures. Poliomyelitis virus strain "M.V." was tested both intracerebrally and intra-ocularly in rhesus monkeys with the results shown in Table IV.

TABLE IV.—*Action of V.I.A. on Poliomyelitis Virus.*

Agent.	Route of inoculation.	Virus dilution.	
		Undiluted.	1/10.
V.I.A.	Intracerebral	10, 11	14, S.
Saline	,,	..	8, 10
V.I.A.	Intra-ocular	17, 24	30, S.
Saline	,,	..	18, 19

S = Survival without paralysis. Numbers = Typical paralysis on the day shown.

These results show only a barely significant effect, in no way comparable to the action on influenza virus.

Three other viruses showed well-marked susceptibility to the agent, louping ill, herpes and virus B. Louping ill virus was tested in parallel in mice (by intracerebral inoculation) and on the chorio-allantois. Table V shows the results with mixtures left for 5 hours at 36° C., the same mixtures being used both for egg and mouse inoculations.

It will be seen that the degree of inactivation is comparable to that obtained with mouse influenza virus, and that the end-point of inactivation is approximately the same in both test objects.

Herpes virus and virus B. are also inactivated readily, typical figures from experiments on the chorio-allantois being :

Herpes : Reduction to average of 15·3 foci from 17,500 under standard conditions of 5 hours at 36° C.

Virus B. : Reduction from 3200 to average of 1·7 foci.

In some experiments we have obtained inactivation of Rous sarcoma virus, but this virus is not a satisfactory one for titration on the chorio-allantois, and further experiments are needed.

A large number of bacteriophages, including types acting on Gram-negative intestinal bacilli, staphylococci and streptococci, have been tested for susceptibility to V.I.A.—in every case with negative result.

TABLE V.—*Action of V.I.A. on Louping ill Virus.*

Agent.	Test object.	Virus dilution. Undiluted.	1/10.	1/10².	1/10³.	1/10⁴.	1/10⁵.
V.I.A.	Egg	++	3, 4, 9	0, 0, 0	..	..	..
,,	Mouse	..	..	9, S, S	S, S, S	S, S, S	..
Saline	Egg	..	..	..	..	10, 15, 2	1, 1, 0
,,	Mouse	..	..	..	6, 6, 7	7, 7, 8	8, 8, S

Egg titrations : ++ = Semi-confluent lesion. 3, 4, etc. = Number of specific foci on each membrane. 0 = No specific foci.
Mouse titrations : 9, etc. = Number of days before typical symptoms. S = Survived 14 days without symptoms.

TABLE VI.—*Action of Lysozyme containing Fluids on "Melbourne" Influenza Virus in Mice.*

Material.	Virus dilution. Undiluted.	1/10.	1/10².	1/10³.	1/10⁴.	Lysozyme activity. 1/10.	1/10².	1/10³.	1/10⁴.
V.I.A.	0, 0, 0	0, 0, 0	..	..	..	++	++	+	−
Saline	..	..	..	3, 3, 2	2, 2, 0	−	..	..	..
Tears, 1 : 2	4^6, 3, 3	4^5, 3, 2	..	..	..	++	++	++	−
,, 1 : 10	4^3, 4^3, 4^4	4^3, 4^6, 4^4	4^5, 4^6, 3	4^5, 3, 3	..	..	..	..	..
Egg-white, 1 : 10	..	4^4, 4^4, 4^4	4^5, 4^6, 4^7	4^6, 3	..	++	++	++	+
Cat saliva	..	4^5, 4^7, 3	4^7, 4^7, 3	3, 2, 2 4^6, 4^6, 4^7	..	++	++	−	−
					3, 3, 2				

++ = Complete lysis of *M. lysodeikticus* suspension. + = Partial lysis. − = No lysis.

Relationship of Lysozyme to V.I.A.

Early in our experiments it was natural to consider the possibility that the active agent was Fleming's lysozyme, which is known to be present in nasal secretion and, in much larger amount, in human tears. A few tests made with diluted egg-white showed no trace of virucidal activity, and this, taken along with the ease with which susceptible viruses may be grown in the developing egg, was at first regarded as sufficient to eliminate lysozyme completely. The observation of Roberts (1937) that human, cat and egg lysozymes were immunologically distinct, however, suggested that human lysozyme might have properties lacking in egg lysozyme. The following materials were therefore obtained : (1) Standard nasal secretion (V.I.A.), (2) human tears (a pool from one adult and one 7-year-old child), (3) fresh egg-white diluted 1/10 in saline, (4) cat's saliva, obtained during the early stages of ether anæsthesia. After centrifugation and heating to 56° C. the fluids were tested for their content of lysozyme on a saline emulsion of *M. lysodeikticus*, and for V.I.A. against mouse-passage influenza virus. The results in Table VI show at once that despite a relatively high lysozyme content, human tears, cat saliva and egg-white have no significant effect in inactivating influenza virus.

DISCUSSION.

The experiments described leave no doubt that there is an active " virucidal" agent in normal human nasal secretion, but as yet we have obtained little information as to the nature of the agent or its significance (if any) in the natural history of influenza virus infections. As far as we can determine from examination of the available literature, no such agent has been previously described. The " inhibin " of Dold and Weigmann (1934) which (in saliva) inhibits the growth of diphtheria bacilli, is said not to be present in serous nasal secretion, though some action is shown by nasal mucus (Ignatius, 1936). Amoss and Taylor (1917) described a substance from human nasal washings which irregularly inactivated poliomyelitis virus, and Howitt (1937) provided a limited confirmation of this finding. An examination of the protocols of these experiments leaves us very doubtful as to whether they are sufficient to justify the conclusion that any specific virucidal agent was concerned. Our own experiments with poliomyelitis virus indicate only a slight but probably significant effect, much less than that on influenza virus.

Perhaps the most interesting feature of the results is that the agent is active only against viruses which are inactivated by sodium desoxycholate (Smith, 1939). Smith found that influenza, louping ill and Rous sarcoma viruses were rapidly inactivated by 0·2 per cent. solutions of this agent, while vaccinia, ectromelia and foot-and-mouth disease viruses were insusceptible. We have extended this series, using a final dilution of 1/400 sodium desoxycholate (B.D.H.) and 2 hours' contact at 36° C. Under these conditions we find influenza, herpes, fowl-pox, infectious laryngo-tracheitis, rabbit myxomatosis, pseudorabies and B. viruses to be inactivated, while vaccinia and psittacosis viruses are unaffected. It may be of interest to note that in a

more limited series of experiments we have found that saponin (0·05 per cent. final dilution) inactivates herpes and influenza, but does not affect fowl-pox (in contrast to desoxycholate) and ectromelia viruses. It will be seen that although all the viruses susceptible to V.I.A. are inactivated by desoxycholate, there are numerous exceptions to the converse statement. Nasal V.I.A. is certainly not a bile salt or similar surface active agent. It has no hæmolytic effect, and its action is far slower than that of desoxycholate or saponin.

We consider it most likely that the agent is an enzyme. This is consistent with the absence of action at 0° C., the progressive course at 36° C., and the destruction of activity by boiling. As far as the limited information in the literature can indicate, the agent is not trypsin, which, for instance, acts strongly on pseudorabies virus, but not on swine influenza virus (Merrill, 1936). There was an obvious possibility that the agent was lysozyme, which is known to be present in nasal secretion. Early experiments showed that the standard source of lysozyme, egg-white, had no inactiviatng effect on influenza virus, but in view of the fact that different lysozymes are immunologically distinct (Roberts, 1937), tests were also made with human tears and cat saliva. These experiments gave no indication that the agent was lysozyme, the only possibility remaining being that one of several human substances which can lyse *M. lysodeikticus*, and can therefore be called lysozyme, has also virus inactivating power.

Experiments on the distribution of V.I.A. in other secretions than that from the nose have so far been very limited. There is very little in human or cat saliva. The watery exudate from the nose of young pigs appears to contain a similar substance, but we were unable to work out a fully satisfactory method of obtaining such exudate. Extracts of nasal mucosa from human beings and swine have so far proved almost inactive, but work on these lines is being continued.

Whether this agent plays any part in determining resistance to infection by influenza and similar viruses remains to be determined. The only evidence we have which appears to have any bearing on the matter is in regard to the presence of the agent during the course of a common cold. In the one instance studied, a typical cold followed by a moderately severe pneumococcal infection of the nose, there was no significant difference in the activity of the nasal exudate obtained at different stages of the cold.

SUMMARY.

1. Serous nasal secretion from normal human beings contains an agent (V.I.A.) capable of inactivating the viruses of influenza (all types), louping ill, herpes, B. virus, and probably Rous sarcoma virus.

2. The agent has no action on the viruses of vaccinia, ectromelia, fowl-pox, infectious laryngo-tracheitis, myxomatosis, psittacosis or pseudorabies. There is a slight but significant action on the virus of poliomyelitis.

3. The inactivation of influenza virus by V.I.A. takes place progressively at 36° C. There is practically no action at 0° C.

4. The activity of V.I.A. is almost completely destroyed by boiling for ten minutes.

5. There is no correlation between lysozyme activity and V.I.A.

6. Viruses susceptible to inactivation by V.I.A. are also susceptible to the destructive action of desoxycholate, but several viruses inactivated by desoxycholate are completely resistant to V.I.A.

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THE ADRENALINE CONTENT OF THE SUPRARENAL GLANDS IN DIPHTHERIA INTOXICATION.

C. A. ASHFORD.

From the Department of Physiology, University of Bristol.

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THE occurrence of pathological changes in the suprarenal glands of man and of experimental animals, poisoned with diphtheria toxin, has been known for many years. These lesions include hyperæmia, hæmorrhages, necrosis and foci of small round cell infiltration. Absence of chromaffin tissue has been reported by many workers (for bibliography, see Myers, 1933); and others *e. g.* Ritchie and Bruce (1911) using guinea-pigs, Tscheboksaroff (1911) using dogs, Luksch (1905, 1909) using rabbits, have claimed to show a complete absence or a great reduction in the content of adrenaline in the suprarenal glands during profound toxæmia. In this observation they have sought an explanation of the circulatory collapse. On the other hand, Hannes (1910), Thomas (1911) and Ingier and Schmorl (1911) did not consider that the cause of death could be related to exhaustion of chromaffin tissue. More recently, Corkill (1932) has reported depletion of chromaffin tissue in poisoned rabbits: on the other hand, Myers (1933) demonstrated by biological methods the

presence of adrenaline in the rabbit adrenal during profound toxæmia and showed that the moribund animal would not respond to injected adrenaline. Many of these results were based on histological methods or chemical tests, and where quantitative assay was carried out, the results were often inconclusive or suffered from lack of adequate controls. Thus, Ritchie and Bruce's results were based on a single estimation ; Ingier and Schmorl (1911) investigated a large number of adrenals from human post-mortem material by a chemical method, and their results do not suggest exhaustion or even depletion of the adrenaline load in children with diphtheria ; Elliott's (1914) figures obtained on children, post-mortem for " normals ", and from fatal cases of diphtheria are too few and suffer from the great variability observed in very young children to be in any way conclusive. His results in other conditions led him to conclude that " suprarenal adrenaline is lessened in many infective diseases, but probably not to such a degree as to endanger the circulation ".

The reports in the literature are thus very variable and the experiments to be described are primarily an attempt to obtain more definite information, by comparing the adrenaline content of the suprarenal glands of normal guinea-pigs and of guinea-pigs poisoned with diphtheria toxin.

METHOD.

Adult guinea-pigs weighing between 500 and 700 g. were used throughout. In all cases the animals were killed by a blow, and the two adrenals dissected out as rapidly as possible. Adherent fat and connective tissue were removed and the glands weighed on a torsion balance. They were then ground with a small quantity of fine sand in a chilled mortar with a known volume of a Ringer-Locke solution (usually 6–8 ml.), buffered to pH 4·7 by 0·02 M sodium phosphate, previously cooled to 0° C. The extract was filtered off and kept at 0° C., an aliquot portion being then used for assay. This method gave a clear extract, suitable for intravenous injection into the decapitate preparation. It was not considered advisable to boil the extracts to remove all protein, as some authors have done. The extracts from the toxæmic animals were always pigmented, due to extraction of hæmoglobin.

The pressor activity of the extract (which is assumed to be equal to its adrenaline content) was assayed on the spinal cat by the technique described by Burn (1937), except that Sherrington's (1909) method of decapitation was used. The preparation usually gave a satisfactory submaximal response to 1 to 10 μg. of adrenaline, injected by means of a cannula placed in the left saphenous or femoral vein.

The experimental animals received 2·5 to 5 M.L.D. of diphtheria toxin by intraperitoneal injection. They usually survived this intoxication for 24 to 48 hours and were killed when deeply toxæmic and usually moribund. Animals which died inadvertently were only used for assay if available a few minutes after death, except in the case of No. 13, which had been dead for 1 to 2 hours. In a few cases only one adrenal was used for assay, the other being retained for histological section. In those cases it was considered permissible to calculate the adrenaline content of the two adrenals on the basis of the weights of the organs. The histological findings will be discussed later.

RESULTS.

The values found for 14 normal animals are shown in Table I*a*. It will be seen that there are very wide variations, and the total amounts in the two glands range between 26 and 100 μg. per animal, with a mean of 56·7 μg. The corresponding values for 13 treated animals are shown in Table I*b*. They range from 24·5 to 98 μg. per animal, with a mean value of 43 μg. per animal.

TABLE I.—*Adrenaline Content of the Combined Adrenal Glands of* (a) *Normal, and* (b) *Guinea-pigs after Receiving Lethal Doses of Diphtheria Toxin.*

Number.	(*a*) Normals. μg. per whole glands.	(*b*) Experimentals. μg. per whole glands.	M.L.D. administered.	Remarks.
1	100	27	3·6	Extract made very soon after death.
2	40	98	5·0	Extract made immediately after death.
3	47·5	31·5	5·0	Animal killed when moribund.
4	26	24·5	5·0	,, ,, ,, ,,
5	75	39·4	2·5	Extract made immediately after death.
6	66	35	2·5	Extract made immediately after death.
7	80	50	3·0	Animal killed—very ill.
8	77	40	3·0	,, ,, ,,
9	70	36	3·0	,, just dead.
10	52·5	40	3·0	,, killed when moribund.
11	52·5	41	3·0	,, ,, ,, ,,
12	40	36	3·0	,, ,, ,, ,,
13	30	60	2·5	,, dead 1–2 hours.
14	37·6	..	..	..
Means	56·7	43		

These values have been submitted to statistical examination by the method of Fisher (1938), using the procedure applicable to a small number of samples. The relevant figures are as follows :

Difference between means	= 13·7
Standard error of difference between means .	= 7·8
Hence the value of " t "	= 1·75
Number of degrees of freedom (n) . . .	= 25

From Fisher's table a probability of 0·1 requires a value of " t " of 1·708 and a probability of 0·05 requires a value of 2·060. It is apparent, therefore, that the difference between the means of the figures analysed above cannot be regarded as reaching the conventional level of significance, *i. e.* a probability of 0·05. However, the value of " t " does exceed the 0·1 probability level, and it follows that the analyses suggest that some reduction in adrenaline content may be associated with diphtheritic intoxication, although this is

manifestly small and in no case approaches exhaustion. The difficulty of establishing the exact effect of the toxin lies in the great variability between animals—a factor which has been inadequately considered in most publications.

As already pointed out, the figures found in the literature are few and have not been treated statistically. Although Ingier and Schmorl (1911) made a large number of analyses, it is not possible to examine their results more exactly, since they give only the mean values, which were not suggestive of a reduction in load, and not the individual figures found.

HISTOLOGICAL FINDINGS.

In one series, adrenal tissue obtained from moribund animals, or immediately after death, was fixed in Susa fixative and stained with eosin and hæmatoxylin. In these, hæmorrhage into cortex and medulla, which in some cases had destroyed the structure, and patches of necrosis, were a prominent feature, while slight destruction of the cells and cytoplasm of the cortex was frequently seen. In another series, fixed in formol and stained with Sudan III and Heidenhain's hæmatoxylin for fat, congestion of the vessels of the whole gland was a constant feature, while reduction of lipoid in the cortex was seen in the majority of preparations. A tendency to fatty degeneration was shown by most of the glands, the stain being taken up extensively in "globular" form, as against the more diffuse staining of the normal glands. This difference in staining was particularly marked in one preparation fixed some few hours after the death of the animal, and although post-mortem changes probably added to this degeneration, it is likely that this picture represents a later stage in the pathology then the preparations obtained from animals killed when deeply toxæmic. These findings are very similar to those of Cramer (1928), who states that "diphtheria toxin produces in the guinea-pig adrenal congestion, with small hæmorrhages in the medulla. The cortex shows a disappearance of the lipoid". He gave no results of the use of his osmic acid staining method for adrenaline in this connection. No definite results have been obtained in my use of this method, largely because the adrenal medulla in the guinea-pig is only a narrow strip in the body of the gland and the cortex is relatively very thick (see, for instance, Elliott and Tuckett, 1906). Consequently, although the gland was first cut into slices before exposure to the vapour, consistent penetration into the medulla was not obtained. Unfortunately the mouse and rat, which have been used so successfully by Cramer in other researches, are not susceptible to diphtheria toxin.

Finally, adrenals from two treated animals have been stained by the bichromate method for chromaffin tissue according to Carleton (1938) (some were also stained subsequently with Heidenhain's hæmatoxylin) and, surprisingly, have shown great depletion and almost exhaustion of chromaffin tissue. It is unfortunate that these glands were not derived from animals in which adrenaline was also estimated, but as they were treated in the same way, it is reasonable to assume that adrenaline was present in considerable quantity. Of course, as an index of the amount of adrenaline present, the method is crude, but it has been used extensively, as already noted, and most authors have

reported consistent reduction of chromaffin tissue in diphtheria poisoning. There are, however, conflicting statements. Thus, Hannes (1910) sometimes found considerable reduction of chromaffin tissue in poisoned guinea-pigs, but in more than half of 47 fatal human cases he actually found an increase of chromaffin tissue. Similarly, Thomas (1911) found reduction in only 3 out of 12 fatal human cases, whereas adrenal chromaffin tissue was consistently reduced in scarlet fever and other infectious conditions. It is obvious, therefore, that as an index of adrenaline content, the method is unreliable.

DISCUSSION.

The fact that adrenaline is still present in high and usually normal concentration in deep diphtheritic toxæmia is contrary to the findings of many workers. There is therefore no basis for the theory that adrenaline depletion is responsible for the circulatory collapse, and indeed, as Myers (1933) has pointed out, the theory that adrenaline administration, by raising the blood-pressure, will improve the circulation is a fallacious one. According to Myers the sympathetic nervous system is depressed and later paralysed and the toxæmic animal will not respond to injected adrenaline. (See also Corkill, 1932 ; Corkill and Ochoa, 1934 ; Molinelli, 1927.)

Reduction of lipoid tissue in the guinea-pig adrenal, as found here and reported by Cramer, is a constant effect of diphtheria toxin, and it may be that this effect on the cortex is responsible for the depression of sympathetic action. In human material Elliott (1914) found disappearance of cortical liquid in death resulting from acute febrile infections, including diphtheria. Cramer (1928) using mice and rats under the action of *B. Welchii* and other toxins, showed by histo-chemical methods, that reduction of adrenaline did not necessarily imply exhaustion of the gland ; so long as the cortex was full of lipoid, the medulla was able to regenerate itself. My animals were always killed during deep toxæmia, and in view of the reduction in cortical lipoid it is surprising that the " adrenalotoxic " action was not found here.

The similarity between metabolic disturbances in Addison's disease and diphtheria has been pointed out by Thaddea (1935). Adrenalectomy gives similar results (Britton and Silvette, 1934). These effects are reversed by injection of cortical hormone preparations. Again, Secker (1938) showed that injection of cortical hormone will restore the retraction response of the nictitating membrane in the adrenalectomized cat after stimulation to exhaustion of the cervical sympathetic nerve, thus suggesting a connection between the adrenal cortex secretion and the integrity of the sympathetic nervous system.

Several workers have investigated the possibility that administration of cortical hormone might improve the circulatory and other disturbances in human and experimental diphtheria. Combination with large amounts of vitamin C was usually found to enhance the results, where favourable. Thus Herbrand (1935), Thaddea (1935), Aicham and Bock (1936), and Ebel and Mautner (1936) found increased survival rates in intoxicated guinea-pigs under the combined therapy, as also did Dieckhoff and Laurentius (1936) in rabbits. In clinical trials Bamberger and Wendt (1935) claimed amelioration

of symptoms in 5 out of 8 children; Bamberger and Zell (1936) reported survival of 26 severe cases, which in their opinion would have been fatal otherwise, from 41 children treated by this method. Bernhardt (1936) also reported favourable results, but unfortunately Berger (1937) has failed to repeat the findings on guinea-pigs, and Dieckhoff and Schuler (1938) found no benefit from this therapy in a series of 93 children. It is possible that these conflicting results may be due to the variability of potency (usually not stated) of the extracts used, since the most favourable results were obtained in animals by high dosage, which it is not easily possible to reproduce in man. Obviously no definite conclusions can be drawn from existing reports, though in view of the observed reduction of cortical lipoid, which seems to be related to the cortical hormone, the possibility is not entirely unfeasible.

SUMMARY.

1. The adrenaline content of the suprarenal glands of a series of guinea-pigs rendered deeply toxæmic by injection of diphtheria toxin has been assayed biologically, and the results compared with estimations on a series of normal guinea-pigs.

2. The figures have been submitted to statistical analysis.

3. The difference between the infected and non-infected animals is small, and the existence of a real difference between the two series is not established with a high degree of probability. The possibility of a reduction in infected animals is indicated, but in no case does this approach exhaustion of the adrenaline load.

4. Certain histological findings are reported, and the possible significance of cortical damage discussed.

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NEUTRALIZATION OF "PURIFIED" TUMOUR AGENT SUSPENSIONS BY ANTI-FOWL SERUM.

R. KNOX.

From the Imperial Cancer Research Fund, Mill Hill, London, N.W. 7.

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GYE and Purdy (1931, 1933), working with the filtrable fowl tumours, found that antisera prepared in goats by injections of normal fowl red cells or of normal chick embryo filtrates could completely inactivate not only crude tumour filtrates which contained considerable amounts of fowl protein, but also active tumour-producing material freed as far as possible from fowl protein by adsorption on kaolin followed by elution. Amies (1937) found that tumour agent suspensions freed as far as possible from fowl protein by differential centrifugation were also neutralized by anti-fowl sera prepared in rabbits. Whereas Gye and Purdy's anti-fowl sera prepared in goats neutralized tumour filtrates only when complement was present, Amies' rabbit sera neutralized "purified" tumour agent suspensions both in the presence and in the absence of complement. The experiments described in this paper were designed to answer two questions:

1. Do anti-fowl sera prepared in goats in the way described by Gye and Purdy neutralize "purified" tumour agent suspensions?

2. If so, does such neutralization occur only in the presence of complement, as was found by Gye and Purdy with crude tumour filtrates,

or does it occur also in the absence of complement, as was found by Amies with anti-fowl sera in rabbits ?

EXPERIMENTAL PROCEDURE.

Tumour agent suspensions.

Rous sarcoma No. 1 was used in all experiments except one in which Fujinami's myxosarcoma was used. A 5 or 10 per cent. extract of tumour tissue prepared by repeated freezing and thawing was lightly spun to remove coarse debris. The supernatant fluid was centrifuged in an Ecco Ultima II centrifuge at 8000 r.p.m. for ½–1 hour ; the coarse thick deposit was discarded and the supernatant fluid centrifuged at 15,000 r.p.m. (about 20,000 times gravity) for 1 hour. The deposit from this run was usually slight, but on resuspending to the original volume the fluid was definitely opalescent. The resuspended deposit was centrifuged again at 8000 r.p.m. for ½–1 hour, the deposit discarded and the supernatant fluid centrifuged again at 15,000 r.p.m. for 1 hour. The deposit, which was usually only just visible, was resuspended in a volume of 1·2 c.c., and centrifuged in a Henriot-Huguenard top at a speed of about 30,000 r.p.m. (or nearly 25,000 times gravity). The deposit was finally resuspended in the required volume, which was usually about one-quarter of the original volume of the crude material. In most experiments normal saline containing 1 in 1000 neutralized potassium cyanide was used as the resuspending fluid except for the final deposit, which was resuspended in saline. In other experiments saline was used throughout. Highly active material was obtained by both methods, though no attempt was made to compare the activity of the final material with that of the original crude extract, and large losses of active material occurred in each stage of centrifugation. In three experiments the crude extract was filtered through paper pulp and Berkefeld N filters before centrifuging ; the deposits obtained were, of course, much smaller than when crude unfiltered extracts were used, but even in these experiments highly active material was obtained.

Anti-fowl sera.

Two goats were selected for immunization, and a large sample of blood taken from each and allowed to clot overnight in the ice-chest. The serum was separated, spun free of cells, filtered through Berkefeld N candles, heated at 55°–56° C. for half an hour, and stored in the ice-chest. Goat 1 received subcutaneous injections of 5 per cent. chick embryo extract. This was prepared by grinding embryos 12–14 days old with sand and 5 per cent. salt solution, followed by distilled water to make a final salt concentration of 0·85 per cent. The extract was then centrifuged lightly and the supernatant fluid used for injecting the goat. The doses given were 10, 15, 20, 25, 30, 35 and 50 c.c. at 5-day intervals. Ten days after the last injection the goat was bled, and its serum, prepared in the same way as the normal serum, is referred to as G 1 immune serum. Goat 2 was injected subcutaneously with washed fowl red blood-cells, and although its serum developed neutralizing power, it was not so strong as the serum of Goat 1, and was only used in one or two experiments.

Neutralization tests.

For neutralization tests the following mixtures were made up:

	"Purified" tumour agent suspension (c.c.)	Saline (c.c.).	Complement (c.c.).	Goat serum (heated at 55° C. for ½ hour) (c.c.).	
A	2	..	1	3	Normal serum before
C	2	1	..	3	immunization.
B	2	..	1	3	Serum obtained after
D	2	1	..	3	immunization.

The contents of each tube were thoroughly mixed and incubated at 37° C., usually for 1 hour, in one experiment for ½ hour and in another for 2 hours. The doses injected were 1 c.c., and 4 or 8 birds were used for an experiment. In most experiments mixtures A and B were injected in opposite sides of each bird in one group, and mixtures C and D into opposite sides of each bird in another group. In two experiments neutralizing power with and without complement was compared in the same group of birds. Tables I and II give the details of two experiments, while Table III gives a rough comparison of the average sizes of tumours produced by the different mixtures in each of seven consecutive experiments.

TABLE I.—*Neutralization of "Purified" Tumour Agent Suspensions by Anti-fowl Serum. Mixtures with and without Complement in Separate Groups of Birds.*

		Tumour agent suspension + complement.				Tumour agent suspension + saline.		
		(A.)	(B.)			(C.)	(D.)	
No. of bird.	Days to death.	With normal serum. Right breast.	With G 1 immune serum. Left breast.	No. of bird.	Days to death.	With normal serum. Right breast.	With G 1 immune serum Left breast.
4368	13	+++	tr.	4372	16	+++	+++
4369	23	+++	,,	4373	20	+++	+++
4370	12	+++	—	4374	15	++	+++
4371	63	+	—	4375	11	+++	+++

Relative sizes of tumours indicated by + signs.
tr. = one or two small nodules detectable only post-mortem.

TABLE II.—*Neutralization of "Purified" Tumour Agent Suspensions by Anti-fowl Serum. Mixtures with and without Complement in one Group of Birds.*

		Tumour agent suspension + complement.				Tumour agent suspension + saline.			
			(A.)	(B.)			(C.)	(D.)	
No. of bird.	Days to death.	Site.	With normal serum.	With G 1 immune serum.	Site.	Site.	With normal serum.	With G 1 immune serum.	Site.
4397	12	Right	+++	tr.	Left	Right	++	+++	Left
4398	21	breast	++	—	breast.	leg	++	++	leg.
4399	27	Right	+++	—	Left	Right	++	++	Left
4400	21	leg	+++	+	leg.	breast	++	+±	breast.

RESULTS.

The number of experiments is small, but Table III shows that—

1. Anti-fowl serum in goats has a strong neutralizing power against purified tumour agent suspensions.

2. This neutralization only occurs in the presence of complement.

Comparison of columns A and B shows that the neutralizing effect is due to the immune serum and not to any inhibitory effect of guinea-pig serum, such as Gye and Purdy described (1931), since complement was included in the control mixture with normal serum, which produced large tumours in the opposite sides of the same birds. Comparison of columns B and D and C and D shows that the anti-fowl serum had no neutralizing effect in the absence of

TABLE III.—*Neutralization of "Purified" Tumour Agent Suspensions by Anti-fowl Serum.*

Experiment.	With complement.		Without complement.	
	(A.) Normal serum.	(B.) Immune serum.	(C.) Normal serum.	(D.) Immune serum.
I	+++	+−	+++	+++
II	+++	−	+++	+++
III	+++	−	+++	+++
IV	+++	++	++	+++
V*	+++	+	++	+++
VI	+++	+	++	+++
VII	+++	−	+	+++

Relative sizes of tumours indicated by + signs.

* In Experiment V Fujinami's tumour was used, in all the others Rous Sarcoma No. 1.

complement, and in some experiments the tumours in column D were larger than in column C. This apparent enhancing effect of anti-fowl serum in the absence of complement has been observed by Gye and Purdy (personal communication). In most experiments mixtures containing saline were made as controls to the mixtures containing complement. In two experiments additional control mixtures were made containing an equal volume of guinea-pig serum inactivated by heating at 55° C. for half an hour, but the result was the same as when complement was replaced by saline.

DISCUSSION.

Under the experimental conditions here described "purified" tumour deposits were neutralized by anti-fowl serum in goats, as were the unpurified filtrates used by Gye and Purdy. Neutralization occurred only in the presence of complement, and in this the results are at variance with those of Amies. Two problems arise :

1. What is the explanation of the disagreement as to the need for complement ?

2. To what extent can material prepared by differential centrifugation be regarded as "purified" ?

The disagreement as to the need for complement may perhaps be explained

by differences in the conditions in the two sets of experiments. Amies used rabbit sera which had been stored in the ice-chest for a long time, while the goat sera used in these experiments had been heated at 55° C. for ½ hour to make certain that the complement was inactivated. But the results cannot be strictly compared except by repeating Amies' experimental conditions, that is to say, testing purified tumour agent suspensions with anti-fowl sera prepared in rabbits. It is possible that the difference may be due to the use of a different species of animal for preparing the immune serum, but it is unlikely that this is the whole explanation, for Amies himself found that neutralization of unpurified material by anti-fowl serum in rabbits was most effective in the presence of complement, and only irregular in its absence. Further experiments are being done to investigate the problem again with rabbit sera. The question may be important, for Gye and Purdy found that whereas anti-fowl serum in goats could only neutralize tumour filtrates in the presence of complement, serum prepared against tumour filtrates neutralized in its absence as well as in its presence. This difference was one of the pieces of evidence on which Gye and Purdy based their belief in the existence of at least two antigens in filtrates of bird tumours, one species-specific in nature, and the other not detectable in the normal tissues of the host in which the tumour grew.

The second problem concerns the criteria which it is justifiable to use for the purity of tumour agent suspensions. Amies used as a test for fowl protein a precipitation reaction with the anti-fowl serum used for neutralization, and found that whereas the crude material gave a copious precipitate, his "purified" material gave none. In some of the experiments here described a complement-fixation reaction was used instead of precipitation as a test for fowl protein, and the purified material gave a negative reaction, while the crude material gave strong fixation. But the sensitivity of a serological reaction for fowl protein as for any other antigen depends upon the titre of the antiserum used for detecting it. The complement-fixing titre of the serum used in these experiments was not high, and a negative serological reaction cannot be taken as fair evidence of freedom from fowl protein. It is possible that Amies' material may have been more highly purified than the material used in these experiments, but essentially similar methods of purification were used, and it has been subsequently found with an anti-fowl serum of high complement-fixing titre that material prepared by the method here described gives a positive reaction at all stages of differential centrifugation. The results and their interpretation will be discussed in a separate paper, but for the present it is enough to say that the use of a high titre anti-fowl serum suggests that it may be impossible to obtain highly active tumour-producing material free from all traces of serologically-reactive fowl protein. This does not necessarily mean that all fowl protein which still gives a serological reaction is to be regarded as an impurity due to traces of tissue debris or soluble proteins, which owing to imperfect technical methods cannot be completely removed. It is possible that a fowl element which reacts serologically with high titre anti-fowl serum is actually part of the tumour-producing agent itself, and this seems perhaps the simplest explanation for the neutralization of so-called purified suspensions by anti-fowl serum.

SUMMARY.

" Purified " tumour agent suspensions are neutralized by anti-fowl sera prepared in goats.

Neutralization occurs only in the presence of complement.

The validity of a serological reaction as a test for fowl protein in " purified " suspensions depends upon the titre of the serum used.

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THE NUTRITION OF *C. DIPHTHERIÆ*. PANTOTHENIC ACID AS AN ESSENTIAL GROWTH FACTOR FOR CERTAIN STRAINS OF *C. DIPHTHERIÆ GRAVIS*: THE SYNTHESIS OF SOME PHYSIOLOGICALLY ACTIVE COMPOUNDS BY *C. DIPHTHERIÆ* CULTURES IN SYNTHETIC MEDIA.

W. C. EVANS, W. R. C. HANDLEY* AND F. C. HAPPOLD.†

From the Biochemical Laboratories, Department of Physiology, School of Medicine, Leeds.

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IN the course of our studies on the nutritional requirements of *C. diphtheriæ* types (Evans, Happold and Handley, 1939) it was found that the *mitis* and some of the *gravis* types could be grown in a synthetic medium containing certain amino-acids, lactate and the usual salts, provided that Mueller's three growth factors—pimelic acid, nicotinic acid or amide and β-alanine—were also present. The *intermediate* strains, however, were found to have rather more exacting nutritional requirements, additional minerals and amino-acids being necessary to support growth. The medium we have used to support the growth of the *intermediate* strains is a total amino-acid medium whose constitution is given in the experimental part of this communication (Medium III).

We have since been concerned with the nutrition of some *gravis* strains, which, as reported previously, failed to grow even in Medium III. These exacting *gravis* strains grew readily in bouillon, and addition of a small quantity of a 95 per cent. alcohol-soluble liver extract or aqueous yeast extract to Medium III also permitted good growth. It became obvious from preliminary experiments that an additional growth factor (or factors) were involved.

* Thorp Research Fellow.

† Leverhulme Research Fellow.

For the preparation of active concentrates we have used the alcohol-soluble liver extract, which is readily available as a by-product in the preparation of pernicious anæmia principles.* More active concentrates, however, weight for weight could be obtained from fresh liver (ox or sheep).

The most ready means adopted of concentrating the active material, after various preliminary trials, was the fractionation of the liver extract on the same general lines as those used by Snell, Strong and Peterson (1937) for the concentration of the active principles concerned in the growth of the propionic and lactic acid bacteria. The most striking property of the active fraction for *C. diphtheriæ gravis* was its instability on boiling with dilute acids or alkalis (normal HCl or NaOH for 10–15 min. completely destroyed its activity). Ignorance of this property led to much fruitless work.

At the same time we were testing out a great variety of growth substances which were known to play some part in the nutrition of bacteria. These included the following : hæmatin, yeast cozymase, aneurin, riboflavin, ascorbic acid, glutathione, glutamine, thymine, uracil, guanine, adenine, adenylic acid and adenyl pyrophosphate, creatine, choline, thymus and yeast nucleic acid (including their hydrolysed products), indolyl acetic acid, meso-inositol, a preparation of biotin, and the *sporogenes* growth factor. The results obtained with these were completely negative when added either alone or in various combinations to our basal medium, and using the technique to be described later. It seemed therefore very unlikely that our active factor (or factors) was identical with any of the well-known growth stimulants.

Our attention was, however, finally directed to the similarity in properties of our active fraction and those published for " pantothenic acid " (Williams *et al.*, 1938). Both are susceptible to acids and alkalis, and the similarity is also parallel in that they show a very strong affinity for water, and are very difficult to extract from aqueous solution by organic solvents.

Through the kindness of Prof. R. J. Williams, who sent us a sample of calcium pantothenate, we were able to test and compare its activity with our most active fractions. The results show that pantothenic acid is active in the same way as the liver concentrates, at a very high dilution (1γ pantothenic acid per 10 ml. basal medium gives a growth with the exacting *gravis* strains comparable with that obtained in tissue extract medium).

It was possible to demonstrate that sterile filtrates from easily growing *C. diphtheriæ* strains in Medium III also contained the active growth factor. This presumably indicates that all the *C. diphtheriæ* strains (*mitis*, some *gravis* and *intermediate*) hitherto grown in our synthetic media actually synthesize the growth factor required by the exacting *gravis* strains.

Whilst this work was in progress, a note appeared by Mueller and Klotz (1938) reporting that pantothenic acid could replace β-alanine in the growth of the "Allen " strain of *C. diphtheriæ*. Moreover, these experiments lent support to the view that β-alanine was effective in so far as it helped as an essential structure for the synthesis of pantothenic acid. We have confirmed this finding for all the diphtheria strains capable of growing in Medium III. It appears that the exacting *gravis* strains are not able to synthesize pantothenic

* We gratefully acknowledge the gift of the liver extract from Boots Pure Drug Co., Ltd.

acid from β-alanine, and in this respect are similar to the lactic acid bacteria as recently reported by Snell and Petersen (1938).

Although we are confident that the "*gravis* growth factor" and pantothenic acid are identical, absolute proof will not be forthcoming until a synthetic preparation of the latter is available. Prof. Williams informs us that the calcium pantothenate was 75 per cent. pure; it is unlikely, although not definitely excluded, that the activity is due to a factor other than pantothenic acid.

In the latter part of this paper experiments are described which show that *C. diphtheriæ* strains, when grown in synthetic media, synthesize substances with similar physiological activities to co-enzyme I or II, aneurin and riboflavin. These substances are presumably directly concerned in the metabolism of the diphtheria bacillus.

EXPERIMENTAL.

Technical Details.

The cleaning of apparatus, and the source of our reagents, are described in our previous paper (Evans *et. al.*, 1939).

Strains.

The exacting *gravis* strains used in this work were those unable to grow in Medium III (below). Six such strains were available, namely: Manchester strains MG 4 and MG 5, Bradford strain "Woods", and Hull strains "Gibson", "Haley" and "Douglas". These last three strains had been recently isolated from toxic or fatal cases of diphtheria.

For the work to be described in the latter part of this communication, *mitis*, *gravis* and *intermedius* strains which had been under investigation in this laboratory were used. All the strains were virulent (in the guinea-pig), and typing was carried out by means of the usual fermentative reactions and observations of their characteristic growth in bouillon and on solid media.

Inoculum.

The strains were grown on blood-agar plates for 12–24 hours at 37° C., a few colonies raked off with a cold sterile platinum loop, care being taken not to remove any of the medium, and suspended in 10 ml. sterile saline. The resulting suspension was then centrifuged, the supernatant fluid decanted and the bacteria re-suspended in a similar volume of sterile saline to an approximate turbidity of 10 million organisms per ml. For inoculation, usually one drop (1/20 ml.), but occasionally one loopful (approximately 1/200 ml.), of this washed suspension has been used. When inoculating into synthetic media, a similar bulk of inoculum was plated on solid media and into bouillon for growth comparisons. Purity of cultures was tested for by terminal plating after each experiment.

Medium III.

The inadequate basal medium used for the demonstration of the activity of various liver fractions with the exacting *gravis* strains was Medium III below:

l-Tryptophane	0·1 g.	s *dl*-Serine	0·1 g.
l-Cystine	0·2 g.	s *dl*-Tyrosine	0·1 g.
*s *dl*-Phenylalanine	0·1 g.	s *dl*-Lysine diHCl	0·1 g.
s Glycine	0·5 g.	*l*-Histidine HCl	0·2 g.
s *dl*-Methionine	0·1 g.	*d*-Arginine	0·1 g.
s *dl*-Valine	0·5 g.	*dl*-Proline	0·1 g.
s *dl*-Glutamic acid	2·5 g.	*l*-Oxyproline	0·1 g.
s *dl*-Alanine	0·1 g.	s *dl*-Threonine	0·1 g.
s *dl*-Leucine	0·5 g.	*dl*-Na lactate	15·0 g.
s *dl*-*Iso*leucine	0·1 g.	†Salt mixture C.	20 ml.
s *dl*-*Nor*leucine	0·1 g.	Distilled water to 1 litre.	
s *dl*-Aspartic acid	2·5 g.		

After tubing in 10-ml. lots the following growth factors were added:

Pimelic acid	1γ
Nicotinic acid	10γ
β-alanine	10γ

per 10 ml. of medium.

*s denotes synthetic amino-acids.

† For the composition of salt mixture C see Evans *et al.* (1939). Medium III is similar in composition to a total amino-acid medium previously employed by us.

Chemical Fractionation of Liver.

Preparation of an active fraction.

Freshly minced ox-liver (1 kg.) was heated to 80° C., after standing overnight with water (5 l.) in the ice-chest. Heating was continued for 30 min., with the addition of small quantities of dilute NaOH to keep the pH 7. The liver coagulum was filtered through muslin, and when cold, the yellow cloudy fluid passed through a fluted filter-paper. The clear neutral filtrate was concentrated to a low bulk (200 ml.) *in vacuo* at 40° C., absolute alcohol then being added to a final concentration of 60 per cent. The solution was filtered again, and concentrated to dryness *in vacuo* at 40° C., the brown residue being finally dissolved in 100 ml. distilled water, and sterilized by filtration. Good growth of the exacting *gravis* strains was obtained upon addition of 0·1 ml. of this aqueous liver extract to 5 ml. basal medium. Activity tests with various dilutions of this "standard liver extract" showed that whilst a hundredfold dilution was still active, a thousandfold dilution either gave no growth at all, or a very much delayed one.

For the further concentration of the active *gravis* factor the most fruitful procedure proved to be a method closely following that adopted by Snell *et al.* (1937).

Lead precipitation.

The raw material (200 g. of the 92 per cent. alcohol-soluble liver extract, representing 40 kg. of liver) was dissolved in 1 litre of water, lead sub-acetate (250 g. in 600 ml. water) was added, and the yellow precipitate filtered off and

thoroughly washed by re-suspension in water. The filtrate and washings were brought to pH 8·5 with ammonia, and the alkali lead precipitate removed and washed as previously. This initial lead precipitation removed a considerable amount of relatively inert material (weight of the combined lead precipitates, 155 g.). The filtrate was acidified slightly to pH 6·5 and the lead removed by H_2S.

Fuller's earth adsorption.

The yellow filtrate from above was diluted to 10 litres and treated with fuller's earth (200 g.) with good stirring of the solution for 30 mins. The solid was filtered off and well washed. The activity was still in the solution.

Norite—charcoal adsorption.

The solution (15 l.) was acidified to pH 3·5 and treated with norite (300 g.) with vigorous mechanical stirring for about 2 hours. The charcoal was filtered off, and the solution re-treated with (200 g.) norite. Elution of the active material adsorbed on the charcoal was effected thus :

(*a*) Suspension in 50 per cent. alcohol (1 l.) at 50° C. with continual stirring for about one hour.

(*b*) The precipitate obtained from the first elution was re-eluated in a similar manner, except that the aqueous alcohol was made definitely alkaline with ammonia (pH 8).

(*c*) Final elution was carried out by treating the norite with one litre of a pyridine-methyl alcohol-water mixture (1 : 2 : 1) at room temperature.

The combined neutralized eluates were evaporated *in vacuo* at 30° C., the dry weight being 12 g. of " eluate fraction ". It was active in extremely small dilution (Table I).

At this point we realized the similarity in properties between the activity of the eluate fraction and pantothenic acid, and further attempts at purification were carried out by the methods described by Williams *et al.* (1939). Briefly, the steps employed were : (*a*) Extraction of the aqueous " eluate fraction " in neutral and acid solution (the former extract weighed 3·6 g., whilst the active acid ether extract weighed 4·0 g.). (*b*) The acid ether extract was converted through the brucine salt into the calcium salt, which was obtained as a white varnish (weight 150 mg.).

A comparison of the activity of our calcium salt with the sample sent by Prof. Williams (75 per cent. pure), showed it to be about one-fifth as active, corresponding roughly to a 10 to 15 per cent. pure calcium pantothenate.

Synthesis of Active Factor by mitis *and non-exacting* gravis *Strains ; Effect of Acid and Alkali Hydrolysis ; Effect of the Presence of* β*-alanine in the Medium.*

In Tables II and III the six exacting *gravis* strains were tested, but only one growth result is recorded, since they all behaved in a similar manner. Table II shows that the active factor is synthesized by *mitis* and non-exacting *gravis* strains. Acid and alkali hydrolysis destroys the factor.

TABLE I.—*The Growth Activities of Various Liver Fractions, with the Exacting* gravis *Strains.*

Medium.		Growth (hours).		
		12.	24.	48
Basal (Medium III) (5 ml.)		−	−	−
Ditto + "Standard liver extract" (10 g./100 ml.)	0·1 ml.	+	++	++++
,, ditto	0·01 ml.	±	+	+++
,, ,,	0·001 ml.	−	∓	+
,, + "Eluate fraction" (0·5 g./100 ml.)	0·1 ml.	+	++	+++
,, ditto	0·01 ml.	+	++	+++
,, ,,	0·001 ml.	−	∓	+
,, + Our own "Ca-salt" preparation (5 mg./100 ml.)	0·1 ml.	+	++	+++
,, ditto	0·01 ml.	±	+	++
,, ,,	0·001 ml.	−	−	−
,, + Williams "Ca-salt" (75 per cent. pure) (1 mg./100 ml.)	0·1 ml.	+	++	+++
,, ditto	0·01 ml.	+	++	+++
,, ,,	0·001 ml.	−	∓	∓

(Here and elsewhere the + signs are roughly proportional to the mass of growth. ± represents a trace.)

TABLE II.—*Growth Experiments Showing* (a) *the Presence of the Active Factor in Diphtheria Filtrates Grown in Medium III;* (b) *Inactivation of the Active Substance by Acid and Alkali Hydrolysis.*

Medium.			Growth (hours).		
			12.	24.	48.
Basal (Medium III)			−	−	−
Ditto + diphtheria filtrates grown in Medium III	*mitis*	"P.W. 1058" (0·5 ml.)	+	++	+++
		"Allen" (0·5 ml.)	++	+++	++++
	gravis	"Freeman" (1·0 ml.)	+	++	+++
,, + pantothenic acid (1 γ per 10 ml.)			++	+++	++++
,, + hydrolysed pantothenic acid (*N* HCl (or NaOH) boiling 10 min.)			−	−	−
,, + active eluate fraction (0·1 ml.)			++	+++	++++
,, + hydrolysed eluate fraction			−	−	−
Bouillon			++	+++	++++

TABLE III.—*Experiment to Test whether Pantothenic Acid is Active Irrespective of the Presence of β-alanine.*

Basal media:

I. Total amino-acids + lactate + salts.

II. Ditto + pimelic acid and nicotinic acid.

Medium.	Growth (hours).		
	12.	24.	48.
Basal I	−	−	−
,, II	−	−	−
,, ,, + pantothenic acid	++	+++	++++
,, ,, + β-alanine (Medium III)	−	−	−
,, ,, + pantothenic acid + β-alanine	++	+++	++++
Bouillon	++	+++	++++

The quantities of growth factors used were the same as usual.

From Table III it is apparent that pantothenic acid is completely active irrespective of the presence of β-alanine.

Effect of Pantothenic Acid on Growth of mitis *and Non-exacting* gravis *Strains.*

In view of our clear demonstration that pantothenic acid is active in the growth of exacting *gravis* strains, and in view of the recording (Mueller and Klotz, 1938) of the replacement of β-alanine by pantothenic acid for the "Allen" strain (*mitis*), it was of interest to see if an acceleration of the growth of easily growing *mitis* and *gravis* cultures took place on supplementing medium III with the growth factor. Accordingly an experiment was planned to test this point, using well-washed suspensions for inoculation, and varying the size of inoculum. The washed suspensions were brought to a uniform turbidity corresponding to roughly one million organisms per ml., and two series of growth experiments set up: (*a*) using one drop as inoculum (1/20 ml.), and (*b*) using one platinum loop (approximately 1/200 ml.). Table IV shows the growth recorded according to its magnitude and at various intervals of time.

TABLE IV.

Medium.		Strain.	Growth results in hours. Drop inoculum. 12.	24.	48.	Loop inoculum. 12.	24.	48.
Medium III	*mitis*	"Allen"	∓	+	++	−	±	++
		688	−	±	++	−	±	++
		"Gallagher"	±	+	++	−	±	++
	gravis	MG 11	−	±	+++	−	±	++
		"Freeman"	±	++	+++	±	++	+++
Medium III + 1γ pantothenic acid	*mitis*	"Allen"	+	++	++++	±	++	++++
		688	±	++	++++	+	++	++++
		"Gallagher"	+	+++	++++	±	+++	++++
	gravis	MG 11	+	+++	++++	±	+++	++++
		"Freeman"	++	+++	++++	+	+++	++++
Bouillon	*mitis*	"Allen"	+	++	++++	±	+	+++
		688	+	++	++++	+	++	++++
		"Gallagher"	+	++	++++	+	+++	++++
	gravis	MG 11	+	+++	++++	±	+	+++
		"Freeman"	++	+++	++++	±	+	++++

It is clear from Table IV that pantothenic acid has a profound effect in that the small lag phase present in Medium III is eliminated. With such an active growth factor it is extremely difficult to be certain that traces are not already present in the medium. There is, however, no doubt that the *mitis* and *gravis* strains, which grow readily in Medium III, actually synthesize it, since filtrates prepared at different periods of growth show an increase in concentration of pantothenic acid when tested with the exacting *gravis* strains.

DISCUSSION.

Although pantothenic acid was discovered as a necessary growth principle for various yeast strains (Williams *et al.*, 1933), it is interesting to note that recently evidence of its importance as a general growth factor has been obtained. Thus, in the field of bacterial nutrition it plays a part as an essential growth component for the *Lactobacilli* (Snell *et al.*, 1938) and also for the hæmolytic streptococci (Subbarow *et al.*, 1939; McIlwain, 1939). Its identity with the chick-antidermatitis factor is almost certain (Woolley *et al.*, 1939).

According to the latest work of Williams *et al.* (1939), pantothenic acid is a compound of β-alanine and a dihydroxy-valeric acid, the union being in the form of a peptide link. Its partial synthesis has already been reported, and there is no doubt that a complete chemical synthesis will be accomplished in the near future.

Little is known about its precise physiological function, but there is some evidence that in the growth of yeasts pantothenic acid is concerned with carbohydrate metabolism (Williams *et al.*, 1936).

THE SYNTHESIS OF ANEURIN BY C. DIPHTHERIÆ STRAINS GROWN IN SYNTHETIC MEDIUM.

It has now been well established that certain micro-organisms, *e. g. Phycomyces blakesleeanus*, *Staphylococcus aureus*, the propionic acid bacteria, etc., require the addition of aneurin as one essential growth factor to their respective basal media. The role of aneurin pyrophosphate (co-carboxylase) as a component part of an enzyme system for the intermediate metabolism of carbohydrates has also been adequately demonstrated. It was of interest, therefore, since the diphtheria bacillus did not require aneurin as a growth factor, to investigate whether the vitamin was synthesized by the organism.

To test this point, use was made of the observations of Fildes *et al.* (1936) on the growth requirements of *Staphylococcus aureus*. This organism grows aërobically on a basal medium of various amino-acids, glucose and salts, provided that aneurin and nicotinic amide are added in small amount (Knight, 1937). In the absence of aneurin growth is not supported.

Experimental Conditions.

Numerous strains of *C. diphtheriæ*, both *mitis* and *gravis*, were grown in Medium III. After 4 days' growth at 37° C. the cultures were filtered to obtain the sterile clear filtrates.

The synthetic basal medium for the growth of staphylococci was distributed in 5 ml. lots in sterile tubes.

Two strains of *Staphylococcus aureus*, one kindly provided by Dr. Gordon, and the other from the National Collection of Type Cultures, were used as test organisms. The technique used throughout as regards inoculum was that described by Fildes *et al.* (1936). The growth results with the staphylococci are recorded in Table V.

TABLE V.

Medium.			Growth of staphylococci. 24 hours.
Basal			−
,, + nicotinic amide (1 γ per 10 ml.)			−
,, + nicotinic amide as above + aneurin (10^{-7} *M*)			+++
,, + nicotinic amide + diphtheria filtrates grown in Medium III	*mitis*	"Allen" (0·5 ml.)	+++
		P.W. 1058 (0·5 ml.)	+++
	gravis	"Freeman" (0·5 ml.)	+++
		M.G. 11 (0·5 ml.)	+++
	intermedius	"Shore" (1·0 ml.)	++
		"Tonsil" (1·0 ml.)	+++
,, + nicotinic amide + uninoculated Medium III (0·5 to 1·0 ml.)			−

It is apparent that the culture fluids of *C. diphtheriæ* strains grown in synthetic medium contain appreciable amounts of a substance having vitamin B_1 activity. As has been shown by Knight (1937), this test, although very specific for aneurin, will not distinguish between its components (the pyrimidine and thiazole compounds), aneurin itself and its pyro-phosphate (co-carboxylase) and possibly a B_1-protein complex. Additional evidence was forthcoming by the following procedure:

Five lots (20 ml.) each of diphtheria cultures (4 days) in medium III were sterilized by autoclaving in acid solution (pH 5) and filtered. The pH was adjusted to 4·5 and passed through a column of permutite, previously prepared, according to the directions of Melnick and Field (1939). Vitamin B_1, if present, is completely absorbed under these conditions. Elution was carried out with 5 ml. acidulated KCl solution (for details of procedure, see the paper quoted). The eluate when tested for the colour reactions of aneurin gave the blue fluorescence characteristic of the thiochrome reaction (Barger *et al.*, 1935), and also the purple-red coloration with diazotized *p*-amino-acetophenone (Prebula and McCollum, 1939).

No attempt was made in these experiments to estimate the aneurin contents of the bacterial cells or the supernatant fluid.

It appears almost certain from these experiments that *C. diphtheriæ* strains grown in synthetic media synthesize aneurin and co-carboxylase. Whether the vitamin plays a similar role in the metabolism of *C. diphtheriæ* as it does in the pyruvate-oxidation system of staphylococci (Hills, 1938) is not yet known.

THE SYNTHESIS OF CO-ENZYME I OR II BY C. DIPHTHERIÆ STRAINS IN SYNTHETIC MEDIUM.

It has been shown by Lwoff and Lwoff (1937) that cozymase (co-enzyme I) or Warburg's co-enzyme II can replace and is presumably identical with the active "V" factor required by *Hæmophilus influenzæ* or *parainfluenzæ*. The supplying of the components of these co-enzymes (I and II)—nicotinic amide, ribose, phosphoric acid and adenine—together was not capable of replacing the "V" factor. From such experiments these authors concluded that *H. parainfluenzæ* did not possess the property of combining these components to form the physiologically active respiratory carriers.

Since nicotinic acid or its amide is active as one of the growth factors required by *C. diphtheriæ*, it appeared probable that this compound was necessary as an essential brick for the synthesis of these co-enzymes.

To settle this problem it was first of all demonstrated that yeast co-zymase could completely replace nicotinic acid at a corresponding dilution 1–10 γ per 10 ml. The preparation did not contain free nicotinic amide, which would vitiate this test.

Experimental Details.

Various strains of *C. diphtheriæ* grown in synthetic media containing rather more than the optimal amounts of nicotinic acid (20–100 γ per 10 ml.) were incubated for four days at 37°. These cultures were sterilized by passing through Chamberland candles, and the clear filtrates were tested for the presence of co-enzymes. The inoculum was a 24-hour blood-agar culture of *H. parainfluenzæ* (N.C.T.C. " Fleming " 4101), a few colonies being suspended in sterile saline (10 ml.) and diluted until the turbidity was just visible.

The peptone-glucose-phosphate buffer medium of Lwoff and Lwoff (1937*a*) was used as the basal medium.

Table VI gives the result of growth experiments with the test organism.

TABLE VI.

Medium.			Growth (hours). 12.	48.
Basal (5 ml.)			−	−
,, (5 ml.) + cozymase (10^{-6} *M.*)			++	++++
,, (5 ml.) + synthetic Medium III (0·5 ml.)			−	−
,, (5 ml.) ,, ,, (1·0 ml.)			−	−
Basal + *C. diphtheriæ* filtrates	*mitis*	"Allen" (0·5 ml.)	+++	++++
		P.W. 1058 (0·5 ml.)	++	++++
	gravis	"Freeman" (0·5 ml.)	++	++++
		MG 11 (0·5 ml.)	++	++++
	intermedius	"Shore" (1·0 ml.)	+	+++
		"Tonsil" (1·0 ml.)	+	+++
Basal (5 ml.) + cozymase (10^{-6} *M.*) + synthetic Medium III (1·0 ml.)			++	++++

The results show that growth took place in the presence of the diphtheria filtrates in place of cozymase, indicating that co-enzyme I or II had been synthesized and discharged in small quantities into the surrounding medium.

THE SYNTHESIS OF RIBOFLAVIN BY C. DIPHTHERIÆ STRAINS IN SYNTHETIC MEDIA.

It has recently been demonstrated that the Lactobacilli group of organisms require for growth on a peptone-glucose-salts medium the presence also of minute amounts of riboflavin (Orla-Jensen, 1936), nicotinic amide and pantothenic acid (Snell and Petersen, 1938).

Drs. Snell and Strong (unpublished—private communication) have developed a method for the detection and assay of small amounts of riboflavin on the basis of the above findings. We are deeply indebted to them for the technical details of their method, prior to its publication.

Good growth of diphtheria cultures in synthetic media is almost invariably accompanied by the production of a yellow pigmentation of the medium, which, however, fades on prolonged exposure to light. Since riboflavin is involved as a prosthetic group in enzymic processes intimately concerned in cellular respiration (Warburg's yellow enzyme and, as recently demonstrated, in some dehydrogenases), it appeared likely that the diphtheria bacillus could synthesize its own riboflavin.

Using *Lactobacillus casei* E as test organism, with the basal medium devised by Snell and Strong, it was found possible to prove the presence of compounds with similar activity to riboflavin in sterile filtrates of diphtheria cultures grown in synthetic media.

Experimental Details.

Sterile filtrates of *C. diphtheriæ* cultures, grown without exposure to light, were prepared as previously described.

Basal medium for Lactobacillus casei *E.*

This was as follows :

0·5 per cent. photolysed NaOH-treated peptone.
0·6 per cent. sodium acetate.
1·0 per cent. glucose.
0·01 per cent. cystine.
0·05 per cent. K_2HPO_4.
0·05 per cent. KH_2PO_4.
0·02 per cent. $MgSO_4$ $7H_2O$.
0·001 per cent. NaCl.
0·001 per cent. $FeSO_4$ $7H_2O$.
0·001 per cent. $MnSO_4$ $4H_2O$.

The above basal medium is supplemented with a yeast extract (10 mg. Difco yeast extract per 10 ml. medium ; the extract was rendered free from riboflavin by lead precipitation). This growth factor supplement has recently been found to be replaceable by nicotinic acid and pantothenic acid in concentrations of 1γ per 10 ml. medium.

Inoculum.

The strain of *Lactobacillus* was grown in the above basal medium, to which was added 1γ riboflavin per 10 ml. medium for 24 hours at 37° C. The growth was centrifuged aseptically, the supernatant medium decanted and the bacteria resuspended in 10 ml. sterile saline. One drop of this suspension was used as inoculum.

Growth Results.

These are shown in Table VII.

TABLE VII.

Test organism: *Lactobacillus casei* E.

Medium.		Growth (hours). 12.	48.
Basal		−	−
,, + yeast supplement + riboflavin		++	++++
,, + nicotinic acid + pantothenic acid + riboflavin		++	++++
,, + yeast supplement + Medium III (0·1 to 1·0 ml.)		−	−
,, + yeast supplement + Medium III (0·1 to 1·0 ml.) + riboflavin		++	++++
,, + yeast supplement + diphtheriæ filtrates	*mitis*: "Allen" (0·1 ml./10 ml. medium)	+	+++
	mitis: ,, (0·5 ml./10 ml. ,,)	++	++++
	mitis: P.W. 1058 (0·1 ml./10 ml. medium)	++	++++
	gravis: "Freeman" (0·1 ml./10 ml. medium)	++	++++
	gravis: M.G. 7 (0·5 ml./10 ml. medium)	++	++++

Growth experiments were also carried out with the *Lactobacillus*, omitting the addition of the yeast supplement to the basal medium, but adding increasing amounts of sterile filtrates of three day-growths of diphtheria strains in Medium III. The results obtained are given in Table VIII.

TABLE VIII.

Medium.		Growth (hours). 12.	48.
Basal		−	−
,, + *C. diphtheriæ* filtrates	"Allen" (*mitis*) (0·5 ml.)	±	+
	,, ,, (1·0 ml.)	+	++
	"Freeman" (*gravis*) (1·0 ml.)	∓	+
	,, ,, (2·0 ml.)	+	++++

The results in Table VIII, whilst confirming the synthesis by *C. diphtheriæ* strains of a substance with the activity of riboflavin in synthetic medium, also lend additional support to the presence of pantothenic acid in the diphtheria filtrates, since growth occurs without the addition of the yeast supplement.

SUMMARY.

1. The factor present in tissue extracts, required by certain exacting strains of *C. diphtheriæ gravis* for growth in synthetic medium, has similar properties to and can be replaced by pantothenic acid.

2. *Mitis* and some *gravis* strains grown in a synthetic medium consisting of amino-acids, lactate and salts, together with the three growth factors of Mueller—nicotinic acid, pimelic acid and β-alanine—can synthesize pantothenic acid. Sterile filtrates of these strains grown in this medium support the growth of exacting *gravis* strains.

3. *C. diphtheriæ* strains (both *mitis* and *gravis*) grown in synthetic medium also synthesize substances with physiological activities similar to aneurin, co-enzyme I or II and riboflavin.

We have pleasure in thanking Prof. J. W. McLeod, Drs. H. Mason Leete, J. Gordon and J. M. Naftalin for cultures used in this investigation. Our sincere thanks are due to Prof. R. J. Williams for a sample of calcium pantothenate, and to the Medical Research Council, who have assisted us with a grant for materials.

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STUDIES IN LEAD MOBILIZATION.

S. L. TOMPSETT AND J. N. M. CHALMERS.*

From the Biochemical Laboratory, the Institute of Pathology of the University and Royal Infirmary, Glasgow.

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A REVIEW of the subject of lead poisoning up to 1925 was made by Aub *et alia* (1926) in their monograph, and further investigations in the decade following were reviewed by Aub (1935). Although the analytical methods described in the monograph have been shown to be somewhat inaccurate, considerable interest was aroused in the biological importance of lead.

Using a new sensitive and accurate method for the estimation of lead in human tissues and excreta (Tompsett and Anderson, 1935), lead can be detected in the tissues of persons with no history of exposure to lead other than the " normal " hazard. The " normal " content of lead in human tissues is derived presumably from the inhalation of dust containing lead, and from the consumption of food and water containing traces of lead. The question of the

* McCunn Scholar.

occurrence of lead in foodstuffs has been reviewed by Monier-Williams (1938), and that of lead in drinking-water by Ingleson (1934).

The concentration of lead varies in the different tissues, the highest concentrations being found in the bones, especially the long bones, such as the femur and tibia (Lynch, Slater and Osler, 1934; Tompsett and Anderson, 1935; Tompsett, 1936). Aub and his co-workers believe that lead is stored in the skeleton exerting no toxic action, while the lead present in the soft tissues and circulation is solely responsible for the symptoms and toxic episodes in plumbism.

Consequently the condition of a person who has absorbed large quantities of lead depends entirely on the relative distribution of lead between the soft tissues and the skeleton. Aub and his colleagues have shown clinically that the toxic episodes in plumbism are relieved quickly by high calcium therapy, and that the clinical improvement is accompanied by a decrease in the amount of lead excreted. This suggests that lead is being laid down in the bones. The clinical side of this work has been confirmed by Belknap (1929).

Two different views are held regarding the treatment of plumbism; in one a high calcium intake is necessary in order to keep the lead locked in the bones; the other favours a process of de-leading, as it is considered that a large store of lead in the bones is a potential danger, and in metabolic disturbances is liable to be mobilized and pass into the soft tissues and circulation. Aub (1935) supports the second view.

From a study of agents likely to produce mobilization and increased excretion of lead, it was found that sodium bicarbonate, potassium iodide and a low calcium intake with or without acidosis-producing substances were effective (Aub, Fairhall, Minot and Retznikoff, 1926). Similar results were obtained with parathormone in conjunction with a low calcium diet (Hunter and Aub, 1927). Litzner, Weyrauch and Barth (1931) confirmed the efficiency of sodium bicarbonate, but not of acidosis-producing substances, the conclusions being based upon the effect of the agent upon the urinary excretion of lead whereas those of Aub were based upon the total excretion.

Lead is present in normal human blood in amounts ranging between 40–60 μg. Pb per 100 ml. (Tompsett and Anderson, 1935). Fifty individuals in the Glasgow area with no history of lead exposure other than the normal hazard were found to contain 30–80 μg. Pb per 100 ml. blood, with a mean of 54 μg. Pb per 100 ml. blood (Chalmers, 1939). Somewhat similar results have been reported by other workers (Blumberg and Scott, 1935; Teisinger, 1936; Teager and Schmitt, 1937; Willoughby and Wilkins, 1938; Kehoe, Thamann and Cholak, 1935).

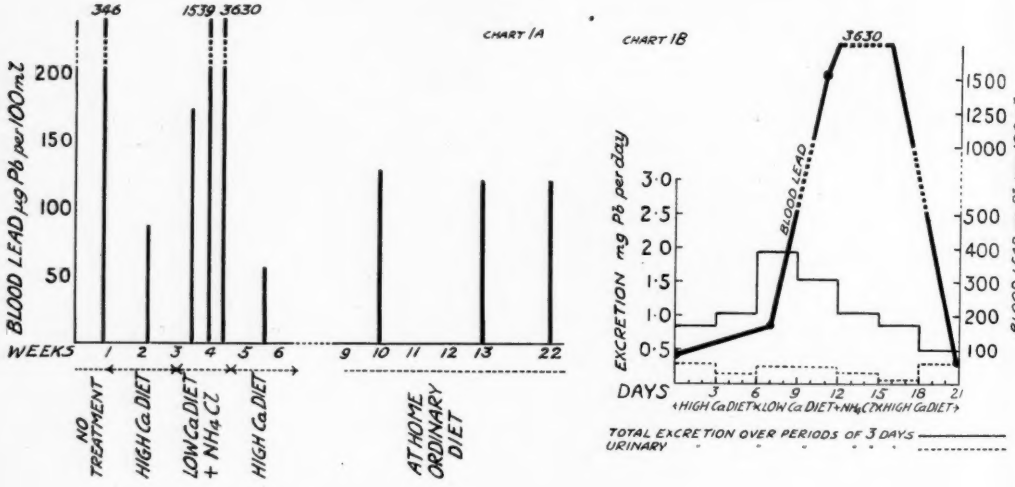
In 1938 we published the results of investigations carried out on a case of lead poisoning produced by the oral administration of lead acetate. It was found that the level of blood lead was affected very markedly by certain changes in diet and by medication. A high calcium intake produced a fall in blood lead, whereas a low calcium diet with or without parathormone produced a rise in blood lead. In a case of lead poisoning of industrial origin, it was noted that potassium iodide produced a marked rise of blood lead, which at the same time was accompanied by a return of the toxic symptoms of plumbism (Tompsett and Anderson, 1935; Chalmers and Tomsett, 1938).

These investigations have been extended to further cases of clinical plumbism

and increased lead absorption, with special reference to the effects of changes of diet and of medication upon the level of the blood lead. In two cases the rate of excretion of lead was studied over a period. The methods of analysis were those of Tompsett and Anderson (1935) and of Tompsett (1939*a*).

The low calcium diets employed contained 0·13 g. Ca per day, and the high calcium diets contained 2·7 g. Ca per day. In some cases the high calcium diet was supplemented by daily intramuscular injections of 10 ml. of a 5 per cent. solution of calcium gluconate. To produce acidosis, ammonium chloride was given by mouth in doses of 1 g. four times per day.

Clinical and hæmatological investigations were carried out at the same time, but the results of these are being recorded elsewhere.



Case 1.—Lead burner (battery works). Male, 29 years. Lead poisoning. The investigations were carried out over an extended period. Following high calcium intake for 14 days, the patient was placed on low calcium diet and ammonium chloride for 9 days. Following this, high calcium diet was maintained. During the beginning of the last period injections of calcium gluconate were given. During the period of medication with ammonium chloride, and for 6 days before and after it, the excreta were collected in periods of 3 days and their lead content estimated. The high calcium diet was maintained for a long period.

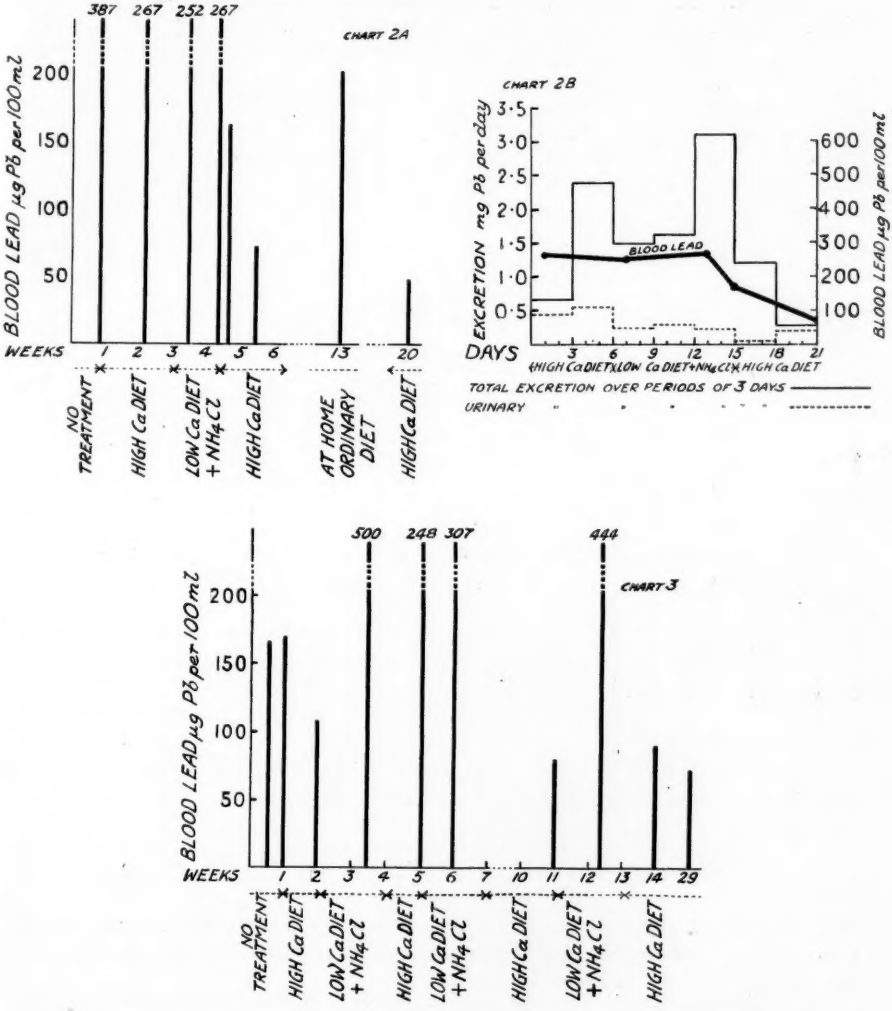
The results obtained are shown in Charts 1*a* and 1*b*.

Case 2.—Red-lead worker. Male, 32 years. Lead poisoning. This patient was treated similarly to Case 1. The results obtained are shown in Charts 2*a* and 2*b*.

Case 3.—White-lead worker. Male, 66 years. Lead poisoning. This patient was placed on alternate periods of high calcium intake and low calcium

intake with ammonium chloride. Finally he was maintained on high calcium diet.

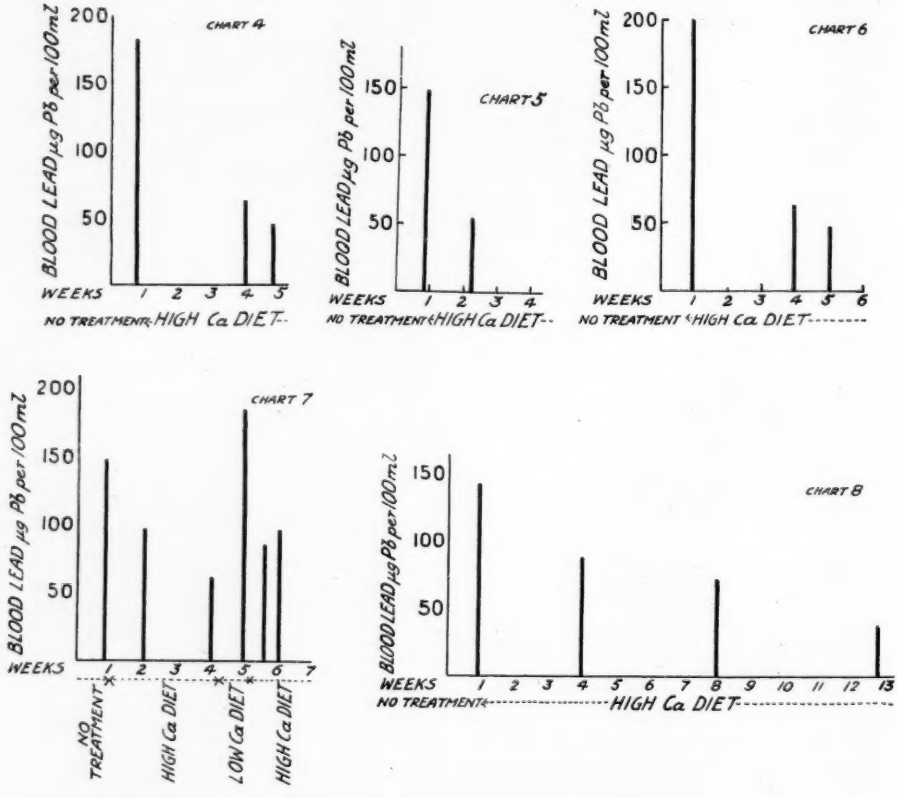
The results are shown in Chart 3.



CASES 4, 5 and 6.—Lead workers with vague symptoms. Males, 25, 33 and 56 years. Increased lead absorption. The blood lead of these patients was determined initially, and then later after a period of high calcium intake. The results obtained are shown in Charts 4, 5 and 6 respectively.

CASE 7.—Lead poisoning due to a lead contaminated drinking-water. Female, 27 years. The effect of high and low calcium intake upon the blood lead was investigated. The results obtained are shown in Chart 7.

CASES 8, 9 and 10.—Increased lead absorption due to a lead-contaminated drinking-water. Males, 34 and 7 years; female, 9 years. The blood lead was

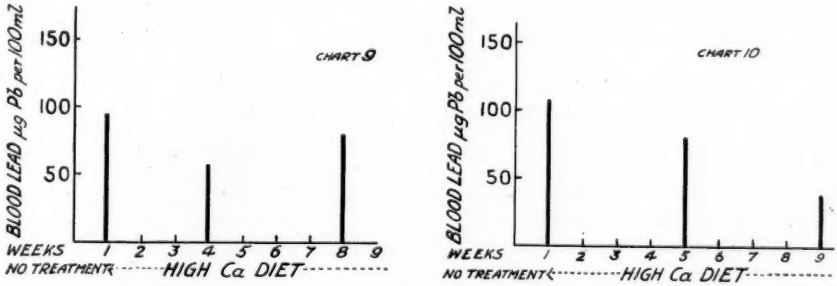


determined initially, and then the patients were maintained on high calcium intake. The blood lead was determined at intervals. The results obtained are shown in Charts 8, 9 and 10 respectively.

The effect of variation in the calcium content of the diet upon lead mobilization as shown by the amount of lead in the circulation is illustrated by Cases 4–10, a high calcium intake being followed quickly by a fall in blood lead. As the treatment in some of the cases lasted over a period of weeks, this could be due in part to loss of lead by excretion. That the calcium content of the diet does exert a powerful influence is best illustrated by Case 7, who

was placed alternately on high and low calcium diet. On high calcium diet the blood lead fell, while on low calcium diet it rose.

The effect of acidosis-producing substances, viz. ammonium chloride, in conjunction with a low calcium diet was studied in Cases 1–3. In Cases 1 and 3 the effect of treatment was to produce marked rises in blood lead, the levels being much higher than those obtained with low calcium diet alone. In Case 1, in which the rate of excretion was studied, the total amount of lead excreted was increased, but not to anything like the same level as the increase in blood lead. In Case 3 high calcium therapy and acidosis therapy were alternated several times. On each occasion responses were obtained as evidenced by the changes in blood lead. In Case 2 the blood lead was hardly affected by ammonium chloride treatment, although the rate of excretion was increased.



This may be explained by the fact that the subject was exposed to lead for a comparatively short period and consequently his store of absorbed lead would be small. Patient 7 died; the lead contents of certain tissues are shown in Table I. When compared with the normal figures for human tissues as obtained by Tompsett and Anderson (1935), it will be seen that all the soft tissues show an increased lead content. The lead content of rib is increased, but that of tibia and femur falls within normal limits.

TABLE I.—*Lead Content of the Tissues of Case* 7.

The results are expressed in mg. Pb per kg. fresh tissue.

Brain	3·08
Liver	7·14
Kidney	4·62
Rib	52·1
Tibia	53·3
Femur	52·1

The changes described in this paper up to now have not been shown experimentally to be due to differences of distribution of lead between soft tissues and skeleton. The following animal experiments were therefore carried out.

Mice were fed with abnormal amounts of lead, and the distribution of lead between soft tissues and skeleton determined under different conditions. It has been shown (Tompsett, 1939*b*) that lead is absorbed from the alimentary

tract, rapidly on a low calcium diet and slowly on a high calcium diet. A series of mice were fed with a low calcium diet of the following composition, together with an added supplement of lead in the form of lead acetate at the rate of 1 mg. Pb. per mouse per day for a period of 14 days.

Low Calcium Diet (Shelling, 1932).

Whole wheat flour	100 g.
Corn starch	325 g.
Casein	100 g.
Wheat gluten	50 g.
Butter	50 g.
Olive oil	40 g.
Sodium chloride	20 g.
Potassium chloride	15 g.

(2·5 g. per mouse per day.)

At the end of this period they were placed on the diet, less lead, for a period of 3 days to remove unabsorbed lead from their alimentary tracts. A third of their number were killed (A). The rest were placed on a high calcium diet for 7 days. This was affected by the addition of 0·5 g. of calcium glycerophosphate to the low calcium diet.

At the end of this period one half of the remainder were killed at once (B), and the others were placed on the low calcium diet for a further period of 7 days before being killed (C). In each animal the skeleton was separated from the soft tissues and the lead content of each determined. The results are shown in Table II.

TABLE II.

The results are expressed in mg. Pb.

A. *On low calcium diet alone.*

	Mouse No. 1.	Mouse No. 2.	Mouse No. 3.
Lead content of soft tissues	0·154	0·168	0·150
Lead content of skeleton	0·105	0·125	0·100
Total lead	0·259	0·293	0·250
Percentage of lead in skeleton	40·6	42·8	40
Percentage of lead in soft tissues	59·4	57·2	60

B. *Low calcium diet followed by high calcium diet.*

	Mouse No. 4.	Mouse No. 5.	Mouse No. 6.
Lead content of soft tissues	0·077	0·089	0·080
Lead content of skeleton	0·250	0·312	0·282
Total lead	0·327	0·401	0·362
Percentage of lead in skeleton	76·4	77·8	77·9
Percentage of lead in soft tissues	23·6	22·2	22·1

c. *Low calcium diet followed by high calcium diet, followed by low calcium diet.*

	Mouse No. 7.	8.	9.
Lead content of soft tissues	0·077	0·087	0·090
Lead content of skeleton	0·143	0·151	0·178
Total lead	0·220	0·238	0·268
Percentage of lead in skeleton	65	64	62
Percentage of lead in soft tissues	35	36	38

DISCUSSION.

The investigation of human subjects whose tissues contain a large amount of lead has shown the extreme mobility of lead. The low calcium diets with or without ammonium chloride produce almost without exception an increase in blood lead, whereas high calcium diets produce a fall of blood lead, in some cases to almost normal levels. The mobility of lead is shown further by the fact that the processes could be repeated several times in succession. It has been shown that ammonium chloride, as a representative of acidosis-producing substances, is an active mobilizer of lead. This supports the work of Aub, Fairhall, Minot and Retznikoff (1926), but contradicts the results of the German workers.

Increase in blood lead does not necessarily parallel increase in lead excretion. Similar results have been obtained by Tompsett and Anderson (1935) in a case of lead poisoning which was treated with potassium iodide. This raises the question of the wisdom of de-leading a patient without adequate laboratory control. The question of the wisdom of de-leading at all must arise, as the process may result in a large influx of lead into the soft tissues and circulation without compensatory increase in excretion.

The technique for the study of agents likely to produce mobilization of lead appears also to require revision. In the past interpretations have been based solely upon changes in part of or in the total excretion of lead. In our view it is necessary in such studies to examine the blood lead in addition to the total lead excretion.

In the diagnosis of plumbism, owing to the influence of diet, a single estimation of blood lead may lead to erroneous results. We would suggest that, in a suspected case, in the event of the blood lead being normal, the estimation should be repeated after a course of low calcium therapy, preferably in conjunction with ammonium chloride. It is also important if conditions permit to examine the lead excretion at the same time.

From an examination of normal tissues lead is present in the highest concentrations in the skeleton, but the distribution is uneven, bones such as the femur and tibia containing much higher concentrations than ribs and vertebræ. In the case described in this paper in which an abnormal amount of lead had been absorbed over a period of two years, the ribs showed a marked increase, but not the femur or tibia. It is probable that under conditions in which abnormal amounts of lead are being absorbed, the more vascular bones such as ribs and vertebræ take the lead in preference to bones of the structure of the femur

and tibia. Similar results were obtained in a case reported by Tompsett and Anderson (1939). The probability is that lead is laid down slowly in the long bones and is more stable, but may be laid down quickly in the vascular bones and is more easily mobilized. It is important, therefore, that in a chemical analysis of tissues in a suspected case of lead poisoning, the vascular bones such as ribs and vertebræ should be examined, since the results obtained from these appear to be as important as those obtained from other tissues.

The animal experiments illustrate particularly well the effect of low and high calcium intake upon the distribution of lead between soft tissues and skeleton. On a low calcium diet lead predominates in the soft tissues, although considerable amounts were found in the skeleton. On high calcium diet lead predominated in the skeleton, but the amount of lead in the soft tissues was not negligible, and was probably capable of producing damage. This point of view would support de-leading.

In all probability imperfections exist in both courses of therapy suggested for the treatment of plumbism. The safest course is naturally preventive.

SUMMARY.

1. The effect of high calcium diet and of low calcium diet with and without ammonium chloride has been studied in cases of lead poisoning and increased lead absorption. High calcium diets produced a fall in blood lead, while low calcium diets with and without ammonium chloride produced a rise in blood lead.

2. There was no parallelism between blood lead level and lead excretion in the two cases examined thus.

3. The effect of high and low calcium diets upon the distribution of lead between soft tissues and skeleton in mice has been studied.

4. The question of treatment in lead poisoning and the aid of the biochemical laboratory in the diagnosis of lead poisoning has been discussed.

In conclusion we wish to record our thanks to Dr. John Gracie, Prof. J. W. McNee, Dr. G. A. Allan, Dr. W. R. Snodgrass and Dr. A. E. Struthers for giving us facilities to study and examine cases in their wards, to Sister Rose for her co-operation with diets, and to Dr. A. B. Anderson for helpful criticism and advice.

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A POLYPEPTIDE RESPONSIBLE FOR SOME OF THE PHENOMENA OF ACUTE INFLAMMATION.

E. S. DUTHIE AND E. CHAIN.

From the Sir William Dunn School of Pathology, Oxford.

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IN 1936 Menkin isolated from various inflammatory exudates a substance which induced in the skin of the rabbit an immediate increase in the permeability of the capillaries. This was demonstrated by the accumulation of trypan blue in skin areas which had been injected with a solution of the substance immediately before the dye was introduced into the blood-stream. The active material was not histamine, since the reactions elicited by histamine in the skin were of a different character, and the active substance had no action on an isolated strip of guinea-pig intestine. In later publications (1938*a*, 1938*b*, 1938*c*) he described a method of purifying the inflammatory principle, which he named "leukotaxine", since sections of the skin areas thirty minutes or less after injection showed that it brought about an emigration of polymorphonuclear leucocytes into these areas. Menkin claimed that he had isolated the substance in crystalline form, and concluded that it was a polypeptide. The purified substance was active in dilutions of 1/100,000 in distilled water. Trypsin digests of serum and of serum albumin were found to contain a substance having a similar action, and were purified in a similar manner.

Since increased capillary permeability and a leucocyte infiltration are always present in the neighbourhood of injured tissue, infected or otherwise, it seemed probable that Menkin's leukotaxine or a similar substance would be present in damaged or partially autolysed preparations of any of the body-tissues. A few preliminary experiments showed that a definite permeability-increasing substance was to be found in extracts of minced liver, kidney and

muscle, with or without autolysis. Bier and Planet (1938) have shown that this is also true in skin. Attempts to purify these extracts by the methods given by Menkin (1938*a*) were unsuccessful, and attention was then directed to enzymatic digests of blood-serum, shown by Menkin (1938*b*) to contain a similar if not identical substance. Attempts to separate the active material by Menkin's method were again unsuccessful, while experiments showed that peptic digests of almost all the proteins, especially the blood proteins, had similar permeability-increasing properties. This made it clear that we were dealing with a general physiological action of protein break-down products, and an attempt was therefore made to isolate in a purer state the product possessing the properties of Menkin's leukotaxine, in order to study more closely its chemical and biological properties. The results of the investigation are presented in the following communication.

PERMEABILITY TEST.

The permeability test used in these experiments was essentially that described by Menkin (1936), but several important modifications were introduced, which made for more consistent results. Rabbits and guinea-pigs were used in the majority of experiments, the former being clipped with scissors immediately before use or shaved a few days previously, and the latter clipped immediately before use with hand clippers. The material to be tested, made approximately isotonic with NaCl, was injected in 0·25 c.c. amounts intradermally into the flanks and back of the animal. Animals possessing a thick dermis were more sensitive and more consistent in their reactions than others, since the active substance was not dispersed so quickly, and it was easier to judge the depth of the injection. For this reason areas close to the limbs and the mid-line were avoided. At least twenty tests could be carried out on the same animal. Immediately before the skin injections approximately 1·25 c.c. per kilo of an isotonic solution of pontamine blue in saline (5 per cent. pontamine blue in 0·6 per cent. NaCl) was injected intravenously. The animals were kept warm, and the material to be injected was brought to body temperature before injection. Since active material in hypertonic solutions of more than 0·25 M was sometimes negative, and active material in solutions of less than 0·1 M gave a slow and uncertain reaction, the solutions were made isotonic in all cases. Distilled water when injected gave at first a blanching effect. After some time when the blood constriction had passed off a slight increase in permeability could be detected, leading to erroneous positive results. This point is emphasized, since Menkin stated that he sometimes injected his material in distilled water, and certain discrepancies between Menkin's results and those reported in this communication may be due to the difference in technique. In reading the reactions it is usually sufficient to smear the animal's skin with liquid paraffin, and watch the development of the staining. In the guinea-pig smaller amounts of the active substance could be detected, and more consistent results were obtained if the animal were killed some forty minutes after the injections and the skin examined from the underside. In the guinea-pig differences in concentration of five-fold and less could be determined with ease.

Leucocyte infiltration was studied in paraffin sections of the injected areas

one hour after the injections were made. Sections were taken down to the panniculus, above which run the main blood-vessels.

FORMATION OF THE ACTIVE SUBSTANCE BY PROTEOLYSIS.

Proteolysis by Pepsin and Trypsin.

At the beginning of the work the experiments were repeated in which Menkin (1938*c*) showed that the active substance was liberated in the digestion of blood-serum with trypsin. A commercial trypsin preparation was used for these experiments, 2 c.c. of serum being incubated with 10 mg. trypsin for 14 hours at 37° C. Since the active trypsin alone showed a positive result, it was found necessary to heat the preparations before injection. Under these circumstances Menkin's results were confirmed, but it was noted that prolonged incubation led to a weakening of the reaction if falling dilutions were tested. Since the active substance was believed to be a polypeptide, it was likely that trypsin might not be the most suitable enzyme to use in its preparation, since its proteolytic action does not stop at the polypeptide stage. Pepsin was therefore substituted, and in a number of preliminary experiments in which rabbit serum was hydrolysed with crystalline pepsin, consistently positive permeability tests were obtained. The experiments were carried out as follows: Two c.c. of rabbit serum was brought to approximately pH 2 by the addition of 2·5 c.c. of 0·3 M HCl, and was incubated with 4 mg. of pepsin for 3 hours at 37° C. The digest was neutralized with 0·3 M NaOH, and when tested gave a positive reaction at dilutions of 1/10 and less. Serum controls diluted with saline gave negative results after 3 hours' incubation, but serum incubated at pH 2, even for short periods without pepsin, gave a faintly positive test, increasing with the time of incubation. In order to obtain more convincing proof of the polypeptide nature of the active substance, the effect of a crystalline pepsin preparation on a crystalline albumin preparation was studied. The results of this experiment and the effect of crystalline pepsin on a number of other proteins are summarized in Tables I and II. The crystalline pepsin used was prepared according to the method of Philpot and Small (1938). The fibrin used was first extracted for several hours in a reflux condenser with 50 per cent. alcohol to remove a water-soluble substance present in commercial fibrin, which alone gives an increased capillary permeability.

TABLE I.—*Permeability-increasing Factor in Protein Digests Tested in Rabbits.*

Protein.	2 per cent. protein incubated 2 hours at 37° C. in NaCl.	2 per cent. protein incubated 2 hours at 37° C. at pH 2.	2 per cent. protein incubated 2 hours at 37° C. at pH 2 + crystalline pepsin 2 mg. per c.c.
Serum albumin	−	±	++
,, globulin	±	+	++
Fibrin	−	−	++
Casein	−	±	±
Egg albumin	−	−	+
Gelatin	−	−	±
Edestin	−	−	+
Histone	+	+	++

TABLE II.—*Permeability-increasing Factor in Protein Digests Tested in Guinea-pigs.*

Protein.	2 per cent. protein incubated 2 hours at 37° C. in NaCl.	2 per cent. protein incubated 2 hours at 37° C. at pH 2.	2 per cent. protein incubated 2 hours at 37° C. at pH 2 + crystalline pepsin 2 mg. per c.c.
Serum albumin	—	—	+
,, globulin	—	—	+
Fibrin	—	—	+
Casein	±	±	+
Egg albumin	—	+	+
Gelatin	—	—	—
Edestin	—	—	±
Histone	±	±	±

In these experiments there was a certain amount of individual variation in both the guinea-pigs and rabbits, and Tables I and II are the summarized results for three animals in either case. In general the reaction was more consistent and more distinct in young animals, and occasionally observed positive responses with undigested proteins were most probably due to allergic reactions (Ramsdell, 1928).

Proteolysis by Papain.

The permeability factor can be produced by the action of papain on the blood proteins, as is shown by the following experiment: An active papain solution was prepared by incubating 22·5 mg. papain (Merck 1/300) in 0·75 c.c. of M/15 phosphate buffer pH 6·3, with 1 c.c. of 3 per cent. neutralized KCN for 1 hour at 37° C. 1 c.c. of the enzyme mixture was added to 5 c.c. of a 4 per cent. solution of the protein in the phosphate buffer, and was incubated for nine hours with shaking. The hydrocyanic acid was removed by warming *in vacuo* at pH 4·7, and the digests were then brought to neutral by the addition of NaOH, and heated for 5 minutes at 100° C. to destroy the papain. Control mixtures were prepared in the same way, but without the addition of the enzyme. The results are given in Table III.

TABLE III.—*Permeability Factor in Papain Digests of Blood Proteins Tested in the Rabbit.*

Protein tested.	Control.	Papain digest.
Serum albumin	—	+
,, globulin	±	+
Fibrin	—	+

Papain solutions in phosphate buffer gave no reactions when tested in the concentrations used in these experiments (0·03 per cent.).

PURIFICATION OF THE PERMEABILITY FACTOR FROM PEPTIC FIBRIN HYDROLYSATES.

With a view to determining which of the blood proteins was the richest source for the purification of the active substance, falling dilutions of peptic hydrolysates of 4 per cent. solutions of the blood proteins were tested. All these digests gave slightly positive permeability tests in dilutions of 1/50, and occasionally at 1/100 in the rabbit. The skin reactions were variable in the rabbit as the end-point was reached, but were more consistent in the guinea-pig. Since blood fibrin is convenient, cheap and commercially available, it was used as a starting material for the purification of the permeability-increasing polypeptide. Parke-Davis pepsin 1/15,000 was used instead of the crystalline product used in the early experiments, and gave equally good results. The purification process was as follows: Pepsin (7 g.) was dissolved in N HCl (22 c.c.) and fibrin (100 g.) added. The mixture was shaken at 37° C. for 4 to 6 hours, and was brought to approximately pH 5·0 and centrifuged. The clear supernatant solution was filtered through a cellophane membrane (Visking casing) enclosed in a silk bag at a pressure of approximately 740 mm. Hg. and at 4° C. The casings are obtainable from J. Crampton & Co., Ltd., Altringham Road, Wythenshawe, Manchester, and the silk used was Swiss bolting cloth silk (double twist), obtainable from J. Stannier, Manchester Wire Works. With a cellophane membrane 2 in. in circumference and 2 ft. in length, about 20 c.c. per hour can be filtered. The content of the filtrate was approximately 2·5 per cent., and was as active as the original digest. It could be stored at 4° C. for a few days without a very great loss in activity. After a number of unsuccessful attempts to separate the active substance from the inactive polypeptides in the filtrate by means of various solvents, and by fractional precipitation with various kations and anions, separation methods based on fractional adsorption were tried. A number of adsorbents were tested at various pHs (calcium carbonate, kieselgur, kaolin, alumina oxide and Fuller's earth). Fuller's earth adsorbed the active substance almost quantitatively, leaving a large amount of inactive material behind, whereas the other adsorbents gave incomplete separation. Considerable difficulty was experienced in eluting the active substance from the adsorbent, and finally the following procedure was adopted: 100 c.c. of the neutralized filtrate was shaken for 10 minutes with 5 g. of Fuller's earth, and centrifuged. The adsorption was then repeated in the same way. After washing with water, the centrifuged deposit was shaken for 30 minutes at 37° C. with 100 c.c. ammonia (concentrated ammonia diluted 1/100). By this means a certain amount of inactive material was eluted, which was then discarded. The deposit was washed with $M/100$ HCl, and then treated for a few minutes with $M/1$ HCl until the gas development ceased. This procedure made possible the subsequent elution of the active material. The Fuller's earth was washed free from HCl, four washings on the centrifuge being usually necessary. The first washings were done at approximately 4000 r.p.m., but the last had to be carried out at approximately 7–8000 r.p.m. The active substance was then eluted by shaking with 100 c.c. of 4 per cent. pyridine for 1½ hours at 37° C. The elution was improved if the solution was brought to 60° C. and stirred at the beginning

of the operation, and repeatedly stirred during the elution. The pyridine solution was then centrifuged at approximately 7000 r.p.m. for at least 30 minutes. The still slightly cloudy solution was then filtered under pressure as before, and was evaporated to about 10 c.c. *in vacuo*. After centrifuging it was dried in a desiccator over phosphorus pentoxide, after freezing *in vacuo*. The total yield was about 80 mg. per 100 c.c. of filtrate.

Activity and Chemical Properties of the Purified Permeability Factor.

The purified material was a light brown, non-hygroscopic substance, which formed a film on evaporation, indicating a high molecular weight. The material was never obtained in crystalline form, and never showed double refraction under the polarizing microscope. It was completely insoluble in any of the ordinary organic solvents (the lower aliphatic alcohols including butyl alcohol, acetone, ether, chloroform, etc.). It was easily soluble, giving a clear, slightly acid solution in water, 60 per cent. alcohol, 50 per cent. acetone and 60 per cent. pyridine. It gave a positive Biuret reaction and was easily dialysable. Tested in 0·85 per cent. saline it gave positive permeability reactions to at least 1/10,000 in the rabbit and 1/100,000 in the guinea-pig (Fig. 1). Sections showed definite leucocyte infiltrations at about ten times these concentrations, the infiltration being more marked in the guinea-pig. Solutions of the permeability factor lost the greater part of their activity within a week at 4° C., but dried and stored in the incubator (37° C.), they were stable for several weeks. The mechanism of this inactivation is unknown.

Inactivation of the Permeability Factor by the Action of Trypsin.

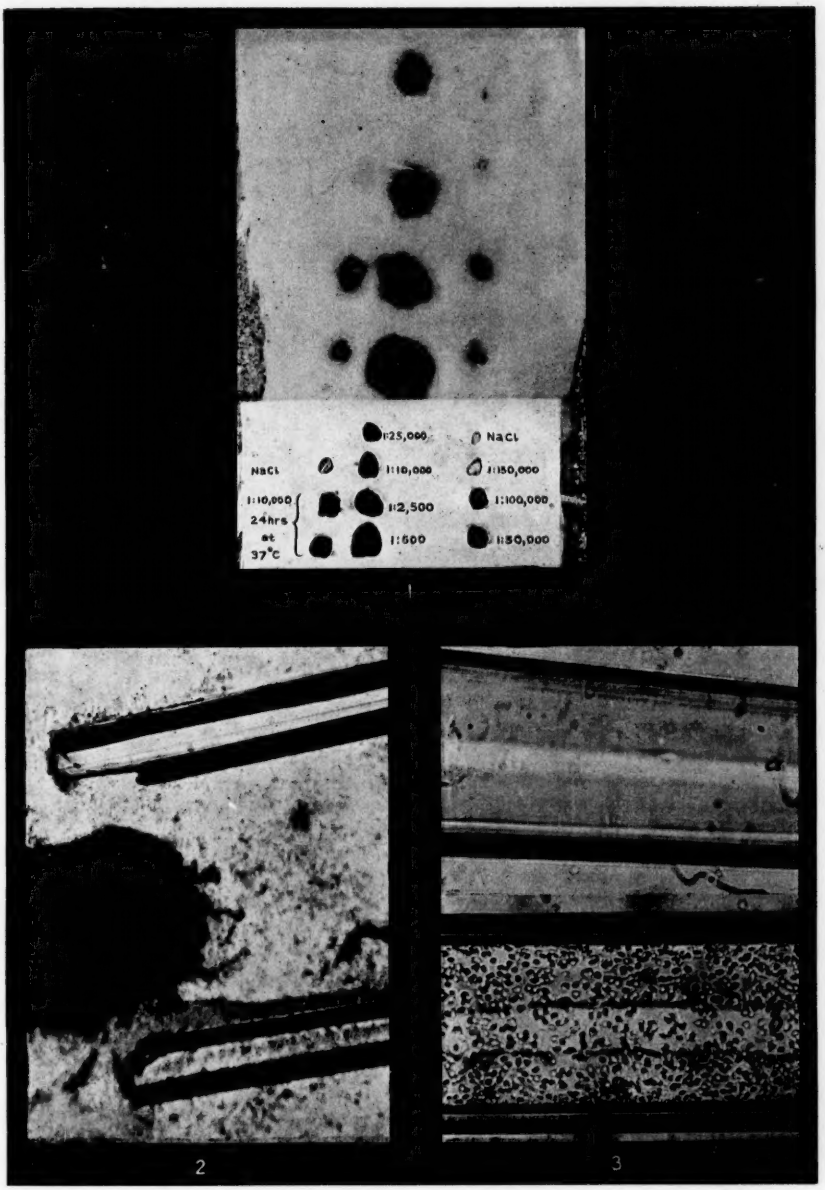
As was to be expected from the original trypsin experiments, the permeability factor was destroyed by trypsin. About 2 mg. of crystalline trypsin was added to 2 c.c. of the filtered and neutralized fibrin hydrolysate. About 1 c.c. of this was brought to pH 8·0, and the remainder was heated to 100° C. to destroy the trypsin. A drop of toluol was added, and both tubes were incubated at 37° C. for 24 hours. The trypsin in the first tube was then inactivated by heating, and the solutions, diluted 1/5 and 1/15, were tested in the rabbit skin. The results in Table IV show that the active material was at least partly destroyed.

FIG. 1.—Undersurface of guinea-pig's skin 45 minutes after testing falling dilutions of the purified polypeptide. The last dilution tested, 1/150,000, gives a slightly stronger reaction than control injections of NaCl.

FIG. 2.—Explant of 17-day-old chick spleen, showing positive leucocyte chemotaxis after 24 hours. The lower quartz capillary tube contained a neutral 1 per cent. solution of the purified polypeptide in physiological saline and fowl plasma, while the upper tube contained the plasma-saline mixture only. Note the massing of leucocytes around and inside the experimental tube.

FIG. 3*a*.—Photograph of a portion of the control capillary tube in the culture shown in Fig. 2 showing complete absence of leucocytes. The photograph was taken at the edge of the zone of migrating cells, and approximately 6 mm. from the inner tip of the tube.

FIG. 3*b*.—Photograph of leucocytes inside the quartz tube containing polypeptide. The section shown is at the same distance from the culture edge and from the tube tip as that in the upper figure.



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UNIV. OF MICH.

TABLE IV.—*Inactivation of the Permeability Factor in Peptic Fibrin Digests by the Action of Trypsin.*

Substance tested.	Permeability test.
Fibrin hydrolysate incubated with crystalline trypsin	—
,, ,, ,, ,, heated trypsin	+
Trypsin in 0·85 per cent. NaCl, heated	—
,, ,, ,, unheated	+

SPECIES SPECIFICITY.

The active permeability factor used for the species specificity experiments was a preparation obtained as follows: A neutralized peptic fibrin hydrolysate was precipitated by the addition of 2 volumes of alcohol, and the precipitate was discarded. The supernatant solution was reduced to about one-third of its volume *in vacuo*, dried in a stream of warm air in a large dish at 47° C., and stored in a desiccator over sulphuric acid. The substance gave a clear solution in water. Two per cent. solutions made isotonic with saline were used in the tests. Histamine acid phosphate 1/1000 neutralized in NaCl and isotonic solutions of NaCl were injected into neighbouring skin areas. The results are given in Table V.

TABLE V.—*Effect of the Permeability Factor from Peptic Fibrin Digests in Various Animals.*

Animal tested.	Permeability test.	
	Histamine 0·1 per cent.	Fibrin hydrolysate 2 per cent.
Dog	Late ring 13·13 mm.	Deep reaction 10·10 mm.
Cat	Deep diffuse 22·22 mm.	Immediate reaction 12·10 mm.
Rabbit	Faint diffuse 20·22 mm.	,, ,, 12·12 ,,
Guinea-pig	,, ,, 18·17 ,,	Immediate deep 12·10 mm.
Rat	Late faint diffuse	Immediate deep reaction.
Mouse	,, ,,	,, ,,

The permeability test followed approximately the same pattern in all the animals. The injection of 0·25 c.c. of the fibrin hydrolysate into the larger animals gave an immediate reaction consisting of a blue area approximately 10 × 10 mm. in size. Histamine phosphate in isotonic saline gave a later more diffuse and usually less deeply stained discoloration, so that in all animals except the cat the skin was never very deeply stained. In the cat not only did the dye stain a more diffuse area around the site of the histamine injection, but the reaction continued for a long period, so that after 30 minutes the histamine-injected areas were deeply stained, and the dye could be seen outlining the lymphatic vessels around them. Experiments in which 0·1 per cent. histamine was added to the fibrin hydrolysate showed in general a permeability test of the histamine type, appearing much earlier and with no diminution in intensity. As described by Menkin (1936), the addition of histamine 1 mg. to 0·25 c.c. of the active material was capable of delaying and partially suppressing the permeability reaction, probably through closure of the smaller skin vessels (Bally, 1929). In none of these animals except the rat and mouse was there any reaction to injections of isotonic saline solutions. In the rat, although

the saline reaction was less than that obtained with the active material or saline, it rendered this animal unsuitable for routine testing.

Using the method described by Lewis (1927), the reaction of the human skin to the purified substance was tested at concentrations of 1/100 and 1/1000, all solutions being in isotonic saline. A drop of each solution was placed on the forearm and the skin pricked through the drop with a sharp needle, after which the drop was removed. Histamine gave the characteristic response—a central red spot followed by a flare some 20 mm. in diameter, and a wheal reaching a maximum size of 4 mm. diameter in about ten minutes. With the 1/100 polypeptide solution the red spot and wheal formed, but there was nothing corresponding to the flare except for a small area of localized reddening around the wheal not more than 5 mm. in diameter over all. The wheal given by the polypeptide grew more slowly than the histamine wheal, but at the end of 15 minutes, when it had reached its maximum size, the wheal, given by the 1 per cent. solution of the polypeptide was approximately equal to that of the 0·1 per cent. histamine. The localized nature of the reaction given by the polypeptide confirmed the impression received from experiments with dye stuff in the skin of various animals.

OTHER PHYSIOLOGICAL ACTIONS.

Action on Heart and Smooth Muscle.

Dr. B. G. Maegraith kindly tested the action of the purified product on the guinea-pig heart and intestine. The injection of 0·5 c.c. of a 2 per cent. solution of the dried purified product (active at 1/80,000 in the guinea-pig skin) had no measurable effect on a guinea-pig heart preparation. The same solution used at a final dilution of 1/4000 had no effect on a strip of guinea-pig intestine. Menkin (1938*a*) also found that his crystalline preparation had no effect on the smooth muscle of the guinea-pig intestine.

Toxicity.

The intravenous injection of 27 mg. of a purified preparation into a 500 g. guinea-pig was without any visible toxic effect. Injections of 5 c.c. of 5 per cent. solutions of the active alcohol-soluble fractions used in the species specificity experiments produced no change in the leucocyte count or in the hæmoglobin level in rabbits over a period of 5 hours.

Vascular Effects.

No effect on the conjunctival blood-vessels could be distinguished following the instillation of 1 per cent. solutions of the active material into the conjunctival sacs of rabbits and guinea-pigs. Histamine at 0·1 and 0·01 per cent. concentrations had a marked vaso-dilator action.

Chemotactic Action.

Chemotactic action in vivo.

In the course of the work a routine examination was made of skin sections of animals killed one hour after the injections were made. In general, protein solutions showed no leucocyte response, while fresh protein digests and

filtrates always produced a leucocyte infiltration into the injected areas. This was either in the nature of an infiltration between the fibres of the dermis, or a very marked diapedesis from the veins above the panniculus. Samples of the purified preparation tested in this way usually showed a definite leucocyte infiltration at strengths of 1/10,000 in the guinea-pig and 1/1000 in the rabbit. The leucocyte response even at these concentrations was variable, and depended on the age of the rabbit and on the regions where the injections were made. Histamine in concentrations of 1/1000 gave no leucocyte infiltration whatever, and this was especially noticeable in the cat, where the depth of dye staining far exceeded that given by the fibrin filtrate, and the latter alone showed leucocyte infiltration.

Chemotactic action in vitro.

For the *in vitro* demonstration of chemotaxis the method given by Silverman (1938), adapted from the original method of Dixon and McCutcheon (1935), was first used. Untreated kaolin was found to possess weak negative chemotactic properties, but after adsorbing a neutralized filtrate of the fibrin hydrolysate it showed a strong positive chemotaxis, comparable to that obtained by Silverman with extracts of skin. Adsorption of comparable concentrations of the purified polypeptide had no effect, although subsequent elution with ammonia showed that the polypeptide had been adsorbed on the kaolin. A very convincing proof of the chemotactic properties of the purified substance in concentrations as low as 1/400 was obtained, however, by Miss R. Schoental (Figs. 2 and 3*a*, *b*), using a modification of the tissue-culture methods described by Grand and Chambers (1936). Two quartz tubes of 0·1 mm. diameter were placed tangentially one on either side of and equidistant from explants of 16-day-old chick spleens, and at distances varying from 1 to 4 mm. in different cultures. One tube was filled with a mixture of fowl plasma and saline, and the other with the same mixture containing the purified polypeptide in various concentrations. In concentrations above 1/400, 12-to 24-hour cultures showed invariably not only a massing of cells around the open end of the tube, but a movement of the cells into the tube, so that at the end of 24 to 48 hours many leucocytes had travelled as much as 10 mm. inside (Figs. 2 and 3). The control tubes showed not only an absence of cell collections around the mouth of the tube, but little if any penetration into the interior. No other substance tested has been found to have such a marked chemotactic effect as the polypeptide.

DISCUSSION.

Since we have obtained polypeptides with permeability-increasing properties from all of the blood proteins after treatment with three different proteolytic enzymes, it is clear that proteolytic processes in the body will liberate similar if not identical polypeptides, and that the result of prolonged proteolysis must be an increase in capillary permeability in the areas affected. Extensive proteolysis is a characteristic feature of inflammation and autolysis, and it is well known that the resulting increased permeability leads to a localization of dyes, bacteria and particulate matter around the inflamed areas (Burrows, 1932). The other characteristic response to prolonged tissue

damage or inflammation is a leucocyte infiltration. It is clear that the permeability-increasing polypeptides have chemotactic properties, since skin injections of both peptic hydrolysates of the blood proteins and of the purified polypeptide led to an early leucocyte infiltration, never present after injections of any of the non-hydrolysed blood proteins and, in addition, the purified polypeptide exerted a strong chemotactic effect when tested *in vitro*. The purified polypeptide therefore reproduces the two most characteristic features of inflammation—increased permeability and leucocyte infiltration. The formation of these polypeptides would explain the well-known chemotactic power of damaged tissue both *in vivo* and *in vitro* (Massart and Bordet, 1891 ; Grand and Chambers, 1936 ; Silverman, 1938). It would also explain the chemotactic effect of pure proteins and of protein-containing solutions *in vivo* (Buchner, 1890 ; Massart and Bordet, 1891 ; Gay, Clark and Linton, 1926 ; Biggart, 1932), since their cleavage will liberate active polypeptides, and of micro-organisms such as the staphylococcus (McCutcheon and Dixon, 1936). These organisms possess strong proteolytic enzymes, whereas the micro-organisms (*e. g.* of the typhoid group), which have no chemotactic properties, are devoid of proteolytic enzymes. It will be interesting to establish the relationship between the chemotactic polypeptide and the polypeptides from the peptic fibrin digests which stimulate cell migration (and growth ?) in tissue culture (Baker and Carrel, 1928*a*, *b*). There is evidence that in tissue injury other chemotactic substances possessing no permeability-increasing properties may be present. If 150 c.c. of 0·85 per cent. NaCl is injected into the peritoneal cavity of a rabbit and a specimen is removed some 24 hours later, it will be found to contain large numbers of leucocytes (de Haan, 1922). Skin injections of the cell-free fluid give a negative permeability test in the rabbit skin, but sections of the skin taken one hour later invariably show a moderate leucocyte infiltration. Similarly the *in vitro* experiments of Grand and Chambers (1936) have shown that various simple sugars and polysaccharides, which do not increase capillary permeability, possess chemotactic properties, and in our own *in vivo* experiments this was true in the case of 1/200 solutions of heated papain and heated commercial trypsin.

Since Lewis (1927) showed the strong similarity existing between the reaction of normal skin to mechanical or other stimulants and to histamine, the part played by histamine in the phenomena of tissue injury and inflammation has been the subject of much speculation. Apart from certain observations which appeared to present exceptions (Rous and Gilding, 1930 ; Goldschmidt and McGlone, 1934), the histamine hypothesis appeared adequate in explaining some of the vascular changes found in tissue injury. It was, however, impossible to demonstrate that histamine had any chemotactic effect when injected locally either in solution (Grant and Wood, 1928 ; Morgan, 1934), in elder pith (Moon, 1938), or in collodion capsules (Bloom, 1922), in order to hinder its too rapid diffusion. Miss R. Schoental has also tested the chemotactic power of histamine phosphate by the *in vitro* method of Grand and Chambers, but was unable to observe positive effects in any of the concentrations tested (1 per cent. to 0·01 per cent.).

Since our investigations did not reveal that the polypeptide isolated by us had any marked vaso-dilator effect, it is unlikely that polypeptides are

responsible for the vascular changes associated with tissue injury, particularly in the early stages. It is, however, clear that whatever may be the role of histamine in the initial stages of inflammation or in mild temporary skin stimulation, polypeptides must be largely responsible for the permanent increase in capillary permeability which always occurs in the later stages of inflammation after proteolysis has begun.

While the biological properties of the purified polypeptide resemble closely those of Menkin's leukotaxine, it is difficult to arrive at any definite conclusions about their chemical relationship, the field of polypeptide biochemistry being still obscure. From Menkin's report it does not seem to us that the polypeptide nature of leukotaxine has been established beyond all doubt. Menkin describes leukotaxine as not completely soluble in water giving a cloudy suspension, and soluble in methyl and butyl alcohol, but not in ethyl alcohol. It is said to crystallize from acetone and butyl alcohol solutions. These are not properties that one would expect a polypeptide to possess. It is, however, possible that the peculiar chemical properties of leukotaxine are due to the presence of impurities. Menkin's scheme of purification involves the extraction of the exudate supernatant after centrifuging with a water-pyridine-acetone mixture, which undoubtedly dissolves most of the lipid material. The solution is evaporated to dryness, and the residue extracted with butyl alcohol. The butyl alcohol extract must contain this lipid material, and as Menkin never mentions any step in his purification process leading to its elimination, we must assume that a considerable amount of lipid material is present in his purified end-product. It is possible that the presence of large amounts of lipid material in butyl alcohol facilitates a solution of polypeptides in this solvent. We note that in his purification of the permeability factor from digested blood-serum, the butyl alcohol extraction was omitted.

Through the kindness of Dr. Menkin we have been able to examine a sample of his purified product. Unfortunately the amount was too small for any extensive chemical investigations, but in agreement with his description the material was a brown, amorphous, hygroscopic substance, which gave a cloudy suspension in distilled water. It had no detectable crystalline structure. nor did it show double refraction under the polarizing microscope. Suspended in NaCl it gave a slight positive permeability reaction at 1/10,000 in the rabbit, and blanching followed by a late reaction when suspended at the same concentration in distilled water. We have unsuccessfully tried to isolate by Menkin's method a permeability-increasing substance from human pus, a saline extract of which gave a positive permeability test in the rabbit. The product which we obtained was very similar in its physical and chemical properties to Menkin's leukotaxine, but had no effect on capillary permeability when tested in the rabbit. Menkin claims that his crystalline material increased capillary permeability in dilutions of 1/100,000 in distilled water. Distilled water in our experience was an unsuitable solvent for skin testing, since the strong blanching reaction was often followed in controls by a dye infiltration into the damaged tissue. In his investigation of chemotaxis Menkin records that cellular migration occurred when a critical concentration of about 1/10,000 to 1/25,000 was reached. This is a lower concentration than that at which we could recognize a leucocyte infiltration in the rabbit with any consistency,

using pure solutions of the polypeptide in NaCl. Unfortunately we did not make sections of the rabbit skin treated with Dr. Menkin's product, so that we are unable to make a direct comparison.

Finally, on the basis of formalin-fixed sections Menkin assigns to leukotaxine a vaso-dilator action. This is hardly in keeping with his demonstration of its almost negligible effect on smooth muscle (Menkin, 1938*a*, 1938*d*), and since he does not appear to have carried out any direct experiments, the claim is not established. Indeed, one of the remarkable points about Menkin's leukotaxine and the polypeptide described in the present paper is the lack of any effect which might be related to the phenomena of shock. Using the polypeptides obtained by the digestion of serum globulin and casein with crystalline pepsin, followed by pressure filtration through a cellophane membrane, we have found that as much as 0·3 g. in 5·0 c.c. of saline may be given intravenously to 300-g. guinea-pigs without effect. Similarly 27 mg. of the purified polypeptide given intravenously was tolerated by a 500-g. guinea-pig. Permeability-increasing polypeptides were present in Witte's peptone when tested, but it does not seem likely that they are responsible for the toxic effects of this product.

SUMMARY.

A polypeptide which produces an increased permeability of the blood-vessels and a leucocyte infiltration in the skin of the rabbit and guinea-pig has been isolated from peptic hydrolysates of blood fibrin. The polypeptide showed strong chemotactic effects on leucocytes *in vitro*.

Polypeptides with similar properties were present in peptic hydrolysates of a number of proteins examined, but were not present in hydrolysates of gelatin.

The polypeptide had no demonstrable effect on smooth muscle and was non-toxic to guinea-pigs.

Its relation to Menkin's leukotaxine and to the phenomena of inflammation is discussed.

We are greatly indebted to Miss R. Schoental for carrying out the *in vitro* chemotactic experiments, to Mr. J. St.L. Philpot, Biochemical Department, Oxford, for samples of crystalline pepsin and for suggesting the method of pressure filtration, and to Prof. Linderstrøm-Lang and Dr. H. Holter, Carlsberg Laboratories, Copenhagen, for the sample of crystalline trypsin used. The work was greatly facilitated by a grant to one of us (E. S. D.) for a technical assistant from the Irish Medical Research Council, and by a part expenses grant from the Government Grants Committee of the Royal Society, to both of whom our best thanks are due.

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THE CHEMICAL COMPOSITION OF THE ACTIVE AGENT OF THE ROUS SARCOMA NO. 1 AND OF SOME RELATED PRODUCTS.

A. POLLARD.

From the Courtauld Institute of Biochemistry and the Bland-Sutton Institute of Pathology, Middlesex Hospital, London, W. 1.

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THE concentration of the tumour-producing agent of the Rous sarcoma No. 1 by methods of centrifugation has been discussed in an earlier communication (Pollard, 1938). The material obtained by the method of fractional centrifugation was found to consist of protein material associated with variable amounts of lipoid substances which were themselves alone shown to be inactive (Pollard and Amies, 1937; Pollard, 1938). The concentrated preparations, when viewed microscopically by dark-ground illumination, were found to consist of apparently homogeneous suspensions of particulate matter, but, as pointed out by Amies (1937), the number of visible particles in such suspensions is greatly in excess of the number required for infection if each particle were capable of giving rise to a tumour. The process of centrifugation merely segregates particles of approximately the same size and density, consequently the final product may contain other material in a similar state of division in addition to inactivated particles of tumour agent and material adsorbed on the surface of the particles themselves. A considerable degree of purification is possible nevertheless, for the tumour agent passes into colloidal suspension more readily than the fine tissue *débris*, and the latter becomes aggregated

during the process of repeated centrifugation and thus can be removed from the more highly dispersed tumour agent. Material adsorbed on the particles themselves may, however, still be present, and make necessary the use of other methods of purification. The work of Stanley (1937) and of Bawden and Pirie (1937) showed that preparations of the tobacco mosaic virus could be freed from inactive protein contaminants by digesting the material with trypsin, and as the tumour agent had been found by Baker and McIntosh (1927) to be resistant to the action of trypsin, its use was applied in the experiments to be described.

The activity of the final product is dependent to a considerable degree on the rapidity with which the preparation can be carried out, for the purified suspensions of the tumour agent readily lose their activity on standing, even at low temperatures, and, as observed by Stern and Duran-Reynals (1939), aggregation of the particles readily occurs. As described in the earlier communication, the tumour-producing agent can be deposited from aqueous extracts of the tissue by means of the Sharples centrifuge, but its quantitative separation from large volumes of extract takes a considerable time. A method for shortening the time of centrifugation was therefore sought. In some earlier experiments preparations of the active material were obtained by acidifying the tumour extracts with acetate buffers (pH 4·5 to 4·7). The resultant precipitate of tumour proteins carried with it the greater part of the tumour-producing activity and the latter could be separated from the inert protein by suspending the precipitate in phosphate buffer at pH 7·4 and adsorbing the protein on alumina in the manner described by Claude (1935). As the process of adsorption was accompanied by serious losses of active material, the final yields were small and the method was accordingly abandoned in favour of centrifugal methods. More recently the method of precipitation has been modified and combined with the method of centrifugation, for as observed by Gye (1925), the tumour agent is more readily deposited in the centrifuge if the extracts are first brought to pH 5 to 5·5. Under such conditions a partial precipitation of the tumour proteins is accompanied by an aggregation of the tumour agent and the latter can be separated from the protein in the combined deposit by the use of trypsin. The method of preparation which has been adopted is briefly summarized below.

PREPARATION OF THE TUMOUR AGENT.

Fresh tumour tissue, which had been ground with sand and rapidly frozen and thawed several times to break up the cells, was extracted with successive portions of ice-cold phosphate buffer (*M*/200 at pH 8) containing hydrogen cyanide (1/10,000) to give a final concentration equivalent to a 5 per cent. tumour extract. The combined extract was freed from gross *débris* by centrifugation at 3500 r.p.m. for ½-hour or by rapid passage through the Sharples centrifuge at 36,000 r.p.m. The clarified extract was then brought to pH 5·5 to 6 by the addition of a phosphate-citrate buffer at pH 5·5. The extract, which had now become more turbid, was passed through the continuous bowl of the Sharples centrifuge at a rate of 2 to 3 litres per hour and the deposit was washed by passing very dilute buffer solution at the same pH through the centrifuge.

The deposit was rubbed off the surface of the metal, suspended in phosphate buffer at pH 8 to redissolve the protein and incubated at 37° C. with trypsin for ½ hour. The enzyme, which had been prepared from the commercial dry trypsin by precipitation with ammonium sulphate (Northrop and Kunitz, 1932), was added in a quantity equivalent to a concentration of about 0·02 per cent. in the solution. A rapid clarification took place and the suspension was then cooled in ice and centrifuged at 5000 r.p.m. for half-an-hour and the supernatant fluid collected. The deposit of fine *débris* was re-suspended in water and centrifuged as before, the process being repeated until the supernatant fluid was no longer opalescent. The combined supernatant fluids were centrifuged for 1 to 1½ hours in an angle centrifuge at 3500 r.p.m. to deposit the tumour agent, and the translucent deposit thus obtained was re-suspended in distilled water and again centrifuged at 5000 r.p.m. for ¼ hour and any deposit removed and washed as before. The supernatant fluids were collected and formed a dense opalescent suspension, which contained no appreciable number of cell fragments or gross particles when viewed under the microscope. An aliquot portion was removed for inoculation in the manner described previously (Pollard, 1938) and the remainder of the material was again deposited in the angle centrifuge. The deposit was coagulated by repeated freezing and thawing and was then washed free of soluble matter by centrifugation. The final washed deposit was dried from the frozen state *in vacuo* for analysis.

The Action of Trypsin.

The incubation with trypsin produced a distinct and rapid clearing of the suspensions, and under the microscope the preparations were seen to consist of particulate matter as before, but the particles showed a greater mobility and less readily aggregated or adhered to the slide. As shown in Table I, the short period of incubation produced no significant change in the infectivity of the material when compared with a control incubated without the enzyme, but longer periods of incubation (1½ hours) produced a slight diminution in the size of the resultant tumours. The chemical changes produced will be discussed later.

TABLE I.—*The Action of Trypsin.*

Experiment number.	Dry weight of material in mg. in a minimal tumour-producing dose.	
	Incubation without trypsin.	Incubation with trypsin.
1	10^{-3}	10^{-3}
2	10^{-3}	10^{-4}
3	10^{-4}	10^{-4}
4	10^{-4}	10^{-3}
5	10^{-3}	10^{-3}

THE FUNCTION OF THE LIPOID FRACTION.

The preparations of the tumour agent obtained by the earlier method of centrifugation contained a large proportion of lipoid substances (40 to 50 per cent.) and the non-lipoid fraction had the nitrogen content of a protein. The

preparations obtained by the method of centrifugation and tryptic digestion also contained lipoid substances in similar or slightly larger amount. In the earlier communications (Pollard and Amies, 1937 ; Pollard, 1938) evidence was produced which suggested that the lipoid material associated with the tumour agent was not concerned with the tumour-producing activity. Extraction of the desiccated tumour tissue with solvents prior to the isolation of the active concentrates removed the greater part of this lipoid material without destroying the tumour-producing activity, but the amount of lipoid material found in the final preparations never fell below about 5 per cent. of the dry weight.

Solvent extraction of the dried and purified tumour agent itself was attempted in order to bring about a more complete removal of the lipoid, but the process of drying the material brought about a great diminution in the activity, due apparently to an irreversible aggregation of the active particles. For when once aggregated the dry material is difficult to redisperse to a colloidal suspension, and the ability of the material to promote the formation of tumours appears closely linked with its ability to penetrate the tissues. This is further suggested by the work of Claude (1939) on the influence of spreading factors on the infectivity of the filterable agents.

It has been found possible to redisperse flocculated suspensions of vaccinia elementary bodies by prolonged mechanical grinding (private communication from Dr. C. R. Amies) ; and this procedure was therefore applied to the redispersion of the aggregated tumour agent. Frozen and dried preparations of the material were ground in a glass tissue-grinder with dilute phosphate buffer at pH 8·5, but although fine and apparently colloidal suspensions were obtained, the activity still fell far short of the original activity and the material was more readily deposited in the centrifuge than were the original suspensions. Preparations which had been dried and then extracted with benzene and ether at 0° C. or at room temperature were treated similarly. A considerable degree of dispersion was attained, but the products were uniformly inactive, although the extraction of the lipoid substances was very incomplete.

To avoid the loss in infectivity accompanying the drying process, aqueous suspensions of the tumour agent were extracted with lipoid solvents. Very highly dispersed and active preparations of the material in dilute phosphate buffer at pH 7·5 or in 20 per cent. ethylene glycol solution at the same pH value were extracted with ether at — 3 to 0° C. either in a continuous extractor or by agitation. A thorough emulsification of the two liquid phases gave a partial extraction of the lipoid material, but the tumour agent was precipitated at the same time and was found inactive. Incomplete emulsification produced no apparent change in the degree of dispersion, but gave very incomplete extraction.

The presence of the lipoid material appears to exert a stabilizing influence on the suspensions, retarding aggregation and possibly forming a protective layer round the particles. It is improbable that the bulk of the lipoid material is bound in any but a loose physical union with the tumour agent, as its general composition is similar to that of the lipoid material in the tumour tissue as a whole, *i. e.* it contains 40 to 50 per cent. of phosphatide material insoluble in acetone together with a mixture of glycerides and sterols. The preparations of

the tumour agent from which the lipoid material had been almost entirely removed, and which were described in a earlier paper (Pollard, 1938), still contained about 5 per cent. of lipoid material. This may be attributed to the incompleteness of the extraction by solvents, or alternatively, the material may be considered to be an integral part of the tumour agent and to be bound more closely with it.

THE ACTION OF FORMALDEHYDE.

Further evidence that the protein fraction of the preparations is intimately concerned with their tumour-producing activity is given by the changes produced by the action of formaldehyde. It has been shown by Ross and Stanley (1938*a*, 1938*b*) that the activity of the tobacco mosaic virus protein runs parallel with the presence of free amino groups, for treatment with formaldehyde reduced both the proportion of free amino groups and the activity of the material. Furthermore, removal of the combined formaldehyde by dialysis or other treatment resulted in an increase in the detectable amino nitrogen and a regeneration of some of the activity.

Similar experiments have been attempted with the tumour agent and the addition of formaldehyde has similarly been found to decrease both the activity and the free amino nitrogen detectable in the preparations. Purified and active preparations of the tumour agent were treated at room temperature for varying periods of time with different concentrations of formaldehyde at pH 7, and the excess formaldehyde was removed by dialysis for 1 to 2 hours in a rotating dialyser at 0 to 5° C. (Lapp, 1936). The activities of the suspensions were compared with those of controls treated similarly, but without the addition of formaldehyde. As shown in Table II, the activity was reduced to 1/10th to 1/100th of the original value in some cases, while in others the activity was entirely removed. The loss of infectivity was accompanied by a small but definite decrease in the amino nitrogen detectable by the Van Slyke apparatus.

The reactivation of the formolized preparations was first attempted by dialysis at different pH values and for varying periods of time (Table II). Dialysis at pH 3 as used by Ross and Stanley was impracticable, although this reaction was found by them to be the most efficacious, for it lies outside the range of stability of the tumour agent. In addition, prolonged dialysis for several days could not be employed, for the great diminution in activity taking place on storage would be sufficient to mask any slight reactivation produced at the same time. Dialysis at pH 5 to 6 for 24 hours at 3° C. produced no apparent increase in the amino nitrogen or in the activity ; on the contrary, the activity of the preparations fell still further. The alternative method of removing the combined formaldehyde by incubation with dimethyl dihydroresorcinol (dimedone) was therefore applied, for although this method was found by Ross and Stanley to be less effective than dialysis at pH 3, it has the advantage of greater rapidity.

Formolized preparations were incubated for short periods with a concentrated solution of dimedone in buffer solution at 30° C. in an atmosphere of nitrogen and were then dialysed against dilute phosphate buffer at 3° C. overnight. The amino nitrogen values showed a significant increase, but no reactivation of the tumour-producing activity was found. After treatment

TABLE II.—*The Action of Formaldehyde.*

Experiment number.	Control.		Inactivation.		Re-activation.		Treatment.
	Titre.	Amino-nitrogen (relative figures).	Titre.	Amino-nitrogen.	Titre.	Amino-nitrogen.	
1	1/100	0·25	1/1	0·18	*nil*	0·17	1 per cent. formaldehyde for ¾ hour, dialysis for 30 hours at pH 7.
2	1/10,000	0·34	1/100	0·24	,,	0·24	1 per cent. formaldehyde for ¾ hour, dialysis for 24 hours at pH 5–6.
3	1/1000	0·20	*nil*	0·15	,,	0·15	1·3 per cent. formaldehyde for ¾ hour, dialysis for 24 hours at pH 5·3.
4	1/1	0·17	,,	0·12	,,	0·15	1 per cent. formaldehyde for ½ hour, incubation with dimedone at pH 5 at 30° C. for ¾ hour and at 5° C. for 15 hours, followed by dialysis for 4 hours at pH 7·5.
5	1/1000	0·15	,,	0·10	,,	0·17	1 per cent. formaldehyde for ½ hour, incubation with dimedone at pH 5 at 30° C. for 1 hour and at 5° C. for 15 hours, followed by dialysis for 3 hours at pH 7·5.
6	1/10	Not estimated	1/1	Not estimated	1/1	Not estimated	0·5 per cent. formaldehyde for ¼ hour, incubation with dimedone at pH 6 at 5° C. for 15 hours.

with dimedone at a lower temperature a partially inactivated preparation still retained some activity, but this was no greater than that of a control left under the same conditions without the reagent. It is therefore possible that the removal of the combined formaldehyde requires conditions which are themselves harmful to the tumour-producing activity, or that the secondary and irreversible changes noted in the case of the tobacco mosaic virus also take place in this instance and to a greater extent.

CHEMICAL ANALYSIS OF THE PREPARATIONS.

The tumour agent obtained by the earlier method of centrifugation (Pollard, 1938) contained, as described above, about 40 per cent. of extractable lipoid material and a non-lipoid fraction with the nitrogen content of a protein (15 to 16 per cent.). The material obtained after tryptic digestion, on the other hand, contained similar amounts of lipoid material, but the non-lipoid fraction gave a lower nitrogen figure, as shown in Table III. It is therefore probable that the earlier preparations contained other protein material which was removed from the later preparations by the treatment with trypsin.

The nitrogen estimations have been carried out by the Kjedahl digestion method followed by titration, or by Nesslerization, according to the amount of material available, the colour in the latter estimations being evaluated by means of a Spekker photo-electric absorptiometer and compared with a standard curve. In addition, estimations of total phosphorus and of carbohydrate have been carried out by means of the absorptiometer; the former by the method described by King (1932) and the latter by the methods of Tillmans and Philippi (1929) and Sørenson and Haugaard (1933) as modified by Hewitt (1937). The carbohydrate has been estimated as glucose, but the true carbohydrate figures may be lower if ribose forms a considerable fraction of the total (*cf.* Bawden and Pirie, 1938), for the purified preparations of the tumour agent give a distinct test for pentose with Bial's reagent.

The non-lipoid fraction of the tumour agent is seen to contain about 13 per cent. of nitrogen and fairly constant proportions of phosphorus and carbohydrate (Table III). Preparations with a similar nitrogen content but with a smaller amount of phosphorus have been described by Claude (1938*a*) who also obtained the material by fractional centrifugation.

NON-FILTERABLE TUMOURS AND BACTERIOPHAGE.

By the courtesy of Prof. J. McIntosh, material derived from several non-filterable tumours has been made available for analysis, in addition to preparations of bacteriophage and of bacteria. Extracts of these tumours, when submitted to fractional centrifugation, gave suspensions of particulate matter which closely resembled those derived from the Rous sarcoma, both in appearance and in general physical behaviour. The analyses of these products are shown in Table III.

The composition of the material from the non-filterable tumours was found to be very similar to that given by the Rous sarcoma, both in the amount of associated lipoid substance and in the composition of the non-lipoid fraction. The bacteria, on the other hand, contained much smaller amounts of lipoid

TABLE III.—*Composition of Purified Preparations.*

Source of material.	Mg. material in minimal infective dose.	Extractable lipoid per cent.	Non-lipoid fraction (as percentages).				
			Nitrogen.	Ash.	Total phosphorus.	Carbohydrate (as glucose).	Carbohydrate/phosphorus.
Rous No. 1 sarcoma	10^{-3}	43	13·5	3·7	0·98	4·8	4·9
,, ,, ,,	10^{-3}	53	13·4	1·7	Not estimated	Not estimated	..
,, ,, ,,	10^{-6}	53	12·7	2·0	1·0	7·0	7·0
,, ,, ,,	Not estimated	46	12·4	2·0	0·8	5·7	7·1
,, ,, ,,	10^{-4}	57	12·6	6·1	1·1	4·5	4·1
* ,, ,, ,,	10^{-5}	39	13·3	2·0	1·03	8·7	8·4
*Tumour 7	..	42	13·3	3·0	1·15	15·7	13·6
*Mellanby tumour	..	39	12·7	0·7	1·1	8·2	7·5
**B. coli* phage	10^{-14}	10·4	13·4	6·0	2·9	25	8·6
**Staph. aureus*	..	0·6	12·3	8·2	2·6	7·9	3·0
* ,, ,,	..	0·3	12·6	7·0	2·5	7·3	2·9
**B. coli*	..	1·6	13·6	5·1	1·9	15	7·9

* Material prepared by Prof. McIntosh.

material, but contained larger amounts of phosphorus. The ratio of carbohydrate to phosphorus in the non-lipoid portion of the Rous agent was found to lie between 5 and 7 in a large number of cases, a ratio not widely different from that found in the tobacco mosaic virus and in the nucleic acid obtainable from it (Bawden and Pirie, 1937). Attempts have been made to separate nucleic acid from the Rous agent by treatment with glacial acetic acid, and a small amount of material richer in phosphorus and in carbohydrate has been obtained (about 6 per cent. of phosphorus and 20 per cent. carbohydrate), but the amounts of material have been insufficient for accurate analysis.

The preparations of *B. coli* phage which have been analysed show a much greater proportion of both phosphorus and carbohydrate, and it has been possible to isolate from the material a fraction insoluble in glacial acetic acid and containing 6 per cent. of phosphorus and 37 per cent. of carbohydrate (estimated as glucose). An impure protein fraction soluble in glacial acetic acid was also obtained, and this contained about 2 per cent. of phosphorus and no detectable carbohydrate. The first fraction gave a very distinct pentose coloration with Bial's reagent and may be assumed to be chiefly nucleic acid originally combined in the bacteriophage as a nucleoprotein. Preparations of *B. coli* phage described by Schlesinger (1934) were found to contain very similar amounts of nitrogen (13·2 per cent.) and a larger amount of phosphorus (3·7 per cent.), but no polysaccharide was detected. The preparations of staphylococcus phage described by Northrop (1938) contained higher proportions of nitrogen, but again they contained very small amounts of carbohydrate. The material prepared by Prof. McIntosh, which possessed a high degree of activity, appears, on the other hand, to be a nucleoprotein associated with a small amount of lipoid material.

DISCUSSION.

The Rous tumour agent certainly appears to contain nucleo-protein, but in much smaller amount than does bacteriophage, and it is probable that the filterable tumour agents are more complex in structure than either bacteriophage or the plant viruses. The analytical figures do not fully account for the total composition of the tumour agent; other undetected substances may be present, or lipoid material may have become insoluble by oxidation and thus contaminate the final product. The enzymatic activity found associated with the Rous tumour agent by Stern and Duran-Reynals (1939) also suggests that the material is either complex in character or is extremely difficult to obtain in a pure condition.

A fraction with physico-chemical properties very similar to those of the Rous tumour agent has been isolated by Claude (1938*b*) from chick embryo tissue, and he has suggested that this may represent a precursor of the infective material isolated from the tumour. Alternatively, the tumour products may be similar inert material with which the actual tumour-producing principle is associated. As this type of product has now been shown to be present also in non-filterable tumours, it may be suggested that such material is concerned with the process of active cell division. It is of interest to note that materials rich in pentose nucleotides have been found by Caspersson and Schultz (1939) to be present in high concentration in rapidly dividing cells of both plant and

animal origin, and their presence in such tissues is considered by the authors to be probably a general phenomenon. The relation between the occurrence of such nuclear material and the malignant change is, however, by no means clear. On the one hand it may be assumed that it is merely a result of the malignant proliferation which has been induced by other factors, while on the other hand the material may be an essential part of the infective agent or may itself be capable of producing the proliferation.

SUMMARY.

The tumour-producing agent of the Rous sarcoma No. 1 can be purified by fractional centrifugation and tryptic digestion. The material contains lipoid material which has a stabilizing influence on the suspensions and protein which is considered to be essential for the activity of the preparations. Treatment with formaldehyde reduces both the activity of the preparations and the detectable amino nitrogen, but the regeneration of the amino nitrogen has not been accompanied by an increase in the tumour-producing activity.

Analyses of the tumour products are discussed together with the analyses of products derived from non-filterable tumours, bacteria and bacteriophage.

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SURVEY OF PAPERS.

In his study of the agglutination reactions of *Cl. tetani*, MacLennan has confirmed and extended the observations of Gunnison. He suggests the use of " O " antisera as an aid to the recognition of aberrant strains, and by this means has identified a new serological type of *Cl. tetani* (p. 371).

Burnet, Lush and Jackson describe an agent (V.I.A.) which is found in human nasal secretion, and which is capable of inactivating influenza and certain other viruses. Experiments with several viruses are detailed. No correlation is found between lysozyme activity and V.I.A. The properties of the agent are discussed and its action contrasted with that of desoxycholate (p. 377).

Ashford has estimated the adrenaline content of the suprarenal glands in diphtheria intoxication to determine whether the content is reduced during the acute stages of diphtheritic toxæmia. Reports on this point, obtained mainly by histological methods on experimental animals and human post-mortem material have been conflicting, and a number of theories of the circulatory collapse have been built up on the assumption of a depletion or exhaustion of the medullary adrenaline.

Guinea-pigs have been used, and the results show that reduction of adrenaline content to any marked extent is not found. Histological findings are reported briefly and the possible significance of cortical damage discussed (p. 385).

Knox confirms findings previously reported by Amies that fowl tumour agent suspensions apparently free from fowl protein are neutralized by anti-fowl serum. There is a discrepancy, however, as to the need for complement in this neutralization. The difficulty of entirely freeing the tumour-producing agent from fowl protein is discussed (p. 391).

Evans, Happold and Handley, continuing their studies on the nutritional requirements of *C. diphtheriæ* types, find that the additional growth factor present in tissue extractives required by certain " gravis " strains, is pantothenic acid. The " mitis " and some " gravis " strains which had previously been grown in chemically defined media synthesize which " gravis growth factor ".

Pantothenic acid is a compound of *B*-alanine and a dihydroxyvaleric acid; according to current literature this substance is identical with the chick-antidermatitis factor, and is one of the " B-complex " vitamins.

The paper also describes experiments which show that *C. diphtheriæ* strains grown in chemically defined media synthesize substances with similar physiological activities to aneurin, riboflavin and " co-enzymes I or II " (p. 396).

Tompsett and Chalmers have shown that in human beings with histories of increased lead absorption, high calcium intake produces a fall in blood lead while low calcium intake, with or without ammonium chloride, produces a rise in blood lead. Changes in blood lead are not necessarily acoompanied by equivalent changes in excreted lead. In mice it has been shown that a high calcium intake produces a transfer of lead from the soft tissues to the skeleton while low calcium intake produces the reverse (p. 408).

Duthie and Chain have isolated a polypeptide from peptic hydrolysates of blood fibrin, intradermal injections of which in high dilutions, caused an immediate increase in the permeability of the skin vessels, together with a leucocyte infiltration. *In vitro* the substance was positively chemotactic to leucocytes. Polypeptides with similar effects were present in hydrolysates of other proteins digested with pepsin, trypsin and papain. It is believed that these polypeptides are responsible for the permeability changes and leucocyte infiltration which occur in inflammation (p. 417).

Pollard has undertaken the preparation of pure suspensions of the tumour-producing agent of the Rous No. 1 sarcoma by methods of centrifugation and has investigated their chemical composition. The preparations contained an inactive lipoid fraction and an active protein fraction containing both phosphorus and carbohydrates, probably as nucleic acid. Products derived by centrifugation from non-filterable tumours were found to be similar in general composition to the Rous agent, although they possessed no infectivity. The nature of such products and their occurrence in the malignant cell are discussed. It is considered that the Rous agent, although consisting mainly of nucleoprotein may be more complex in character than is bacteriophage which has been found to consist almost entirely of nucleoprotein (p. 429).